REMARKS

Claims 109-124 are all the claims pending in the application.

The Examiner is thanked for conducting a telephonic interview on April 27, 2006 in order to try to find language to overcome the 35 U.S.C. § 112 rejections. As a result of the interview all previous claims have been cancelled, and new claims 109-124 are presented for consideration. Also, submitted herewith is a Statement of Substance of Interview, summarizing the interview in more depth.

New claims 109 - 113 are directed to an isolated polypeptide comprising at least amino acids 103-123 of SEQ ID NO:1, wherein serine-118 is replaced by alanine or glycine, and to isolated polypeptides that have various degrees of sequence identiy with SEQ ID NO: 1, but wherein amino acids 103-123 are not changed, other than by replacing serine-118 with alanine or glycine.

Support for these claims is found in canceled claims 89-94 and in the specification at page 76, lines 13-17 and page 45, lines 13-16.

New claims 114 - 118 are directed to an isolated polypeptide comprising at least amino acids 106-132 of SEQ ID NO:1, wherein serine-118 is replaced by alanine or glycine, and to isolated polypeptides that have various degrees of sequence identiy with SEQ ID NO: 1, but wherein amino acids 103-123 are not changed, other than by replacing serine-118 with alanine or glycine.

Support for these claims is found in canceled claims 99-104 and in the specification at page 76, lines 13-17 and page 45, lines 13-16.

New claims 119-124 are directed to an isolated polypeptide comprising (claims 119-121) or consisting of (claims 122-124) SEQ ID NO: 1, wherein serine-118 is replaced by alanine or glycine.

Support for these claims is found in the specification at page 9, lines 4-18 and page 76, lines 13-17.

Accordingly, no new matter is added.

Further, it is believed that the claims so clearly overcome or avoid the rejections below, that no new issue of patentability arises.

Accordingly, entry and consideration of the amendment is requested, respectfully.

I. Claim Rejections Under 35 U.S.C. § 112, First Paragraph - New Matter

At pages 2-4 of the Office Action, claims 80-83, 85-93, 95-103, and 105-108 were rejected under § 112, first paragraph, for failure to meet the written description requirement.

Specifically, the Examiner asserted that the phrase "an amino acid conservative for alanine" is not described in the specification, and, thus, constitutes new matter.

The new claims do not recite the alleged new matter.

Instead, the new claims recite that serine-118 can be replaced with glycine. The specification at page 76, lines 13-17 makes clear that the inventors contemplated that serine-118 could be replaced with glycine.

II. Claim Rejections Under 35 U.S.C. § 112, Second Paragraph - Indefiniteness

At page 4 of the Office Action, claims 81-108 were rejected under 35 U.S.C. § 112, second paragraph.

In particular, the Examiner asserted that recitation of the "position corresponding by sequence alignment with SEQ ID NO:1 to position 118 of SEQ ID NO:1" is indefinite.

The new claims do not use the phrase alleged to be indefinite.

III. Claim Rejections Under 35 U.S.C. § 112, First Paragraph - Written Description

At pages 4-13 of the Office Action, claims 81-108 were rejected under 35 U.S.C. § 112, first paragraph, for failure to meet the written description requirement.

A. Sequence Alignment

The Examiner asserted that the specification discloses the results of sequence alignment, but does not define sequence alignment.

The new claims do not recite the subject matter in terms of sequence alignment.

B. Variant BH3 Domains

The Examiner asserted that the art does not teach which and/or how many amino acids of the BAD BH3 region actually could be substituted, added, or deleted, without affecting the cell death promoting activity of dephosphorylated BAD. Thus, the Examiner concluded that the

polypeptides described in the specification are not representative of the scope of variant polypeptides encompassed in the claims.

The new claims do not recite homology to the BH3 domain.

In this respect, the specification discloses, by analogy to the murine BAD, that *in vitro* cell death promoting activity is observed when serine-118 is replaced with alanine (Example 2, especially pages 76-77) or a non-phosphoralatable mutant serine in a full-length BAD. The specification further demonstrates, again by analogy to the murine BAD, that a polypeptide comprising amino acids 103-123 of SEQ ID NO:1 is sufficient at least to allow binding to Bcl-X_L (Example 2, especially the paragraph bridging pages 76 and 77).

In addition, Moreau et al., J. Biol. Chem., Vol. 278, No. 21, pages 19426-19435 (2003) (copy submitted herewith) shows that amino acids comprising the BH3 domain (108-123) compel cells to undergo apoptosis. The BAD BH3 domain peptide is shown in the Materials and Methods section. See under the title peptides and recombinant proteins, page 19427. The results are shown in Figure 5 b. In the description section it is clearly stated at the bottom of page 19431 (left panel) that "BadBH3 alone . . . induced . . . apoptosis." This is reiterated in the introduction (upper left panel page 19427), wherein it is stated that "these 16 residues contain sufficient information to bind to and functionally antagonize Bcl-XL and to induce specifically Bax/Bax-mediated apoptosis."

Furthermore, the specification suggests that glycine can safely be substituted for serine-118. (page 76, lines 13-17). See also Bordo et al., J. Mol. Biol., Vol. 217, pages 721-729 (1991) (copy submitted herewith), which shows that glycine or alanine can safely be substituted for serine in other proteins. In particular, see Figure 1 (b) and (c) and Figure 2 of Bordo et al., which show that glycine or alanine is a safe substitution for serine, because such substitution provides the least likely possibility of disturbing the protein structure and is most likely to allow probing of structural and functional significance (see, left panel, 2nd paragraph on page 727).

C) Bcl-X_L and Bcl-2 binding

At pages 12 and 13 of the Office Action, the Examiner noted that although the remarks in the November 22 Amendment indicate that the recitation of Bcl-X_L and Bcl-2 binding has been deleted from the claims, these deletions were not made.

The new claims do not recite Bcl-X_L and Bcl-2 binding activity.

IV. Claim Rejections Under 35 U.S.C. § 112, First Paragraph - Enablement

At pages 13 and 14, claims 81-108 were rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement.

The issues raised by the Examiner in the enablement rejection are essentially the same as those raised in the written description rejection, discussed above. Thus, Applicants believe that the response to the written description rejection overcomes the enablement rejection.

V. Rejections Under 35 U.S.C. § 102 - Anticipation

At pages 15 and 16 of the Office Action, claims 81-108 were rejected under 35 U.S.C. § 102(e) as being anticipated by US Patent No. 5,965,703.

Specifically, the Examiner asserted that because the term "the amino acid at the position corresponding by sequence alignment to position 118 of SEQ ID NO: 1" is not defined by the specification and because one cannot determine which amino acid is the reference point for sequence alignment in the claims, any amino acid could correspond by sequence alignment to position 118 of SEQ ID NO:1. Thus, the Examiner concluded that the present claims encompass wild-type human BAD.

The new claims do not use sequence alignment language and clearly indicate that a particular amino acid, serine-118 in a defined sequence, SEQ ID NO:1 or residues 103-123 or 106-132 of SEQ ID NO:1, is replaced.

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

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Suggestions for "Safe" Residue Substitutions in Site-directed Mutagenesis

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The conserved topological structure observed in various molecular families such as globins or cytochromes c allows structural equivalencing of residues in every homologous structure and defines in a coherent way a global alignment in each sequence family. A search was performed for equivalent residue pairs in various topological families that were buried in protein cores or exposed at the protein surface and that had mutated but maintained similar unmutated environments. Amino acid residues with atoms in contact with the mutated residue pairs defined the environment. Matrices of preferred amino acid exchanges were then constructed and preferred or avoided amino acid substitutions deduced. Given the conserved atomic neighborhoods, such natural *in vivo* substitutions are subject to similar constraints as point mutations performed in site-directed mutagenesis experiments. The exchange matrices should provide guidelines for "safe" amino acid substitutions least likely to disturb the protein structure, either locally or in its overall folding pathway, and most likely to allow probing of the structural and functional significance of the substituted site.

1. Introduction

Site-directed mutagenesis has become a very important and yet facile tool to explore the structural and functional significance of particular residues within proteins (for example, see Knowles, 1987; Shaw, 1987; Gruetter *et al.*, 1987). A typical experiment would involve substitutions of an amino acid thought to be essential for catalysis and then assaying the resultant variant for activity. It is central to the success of these experiments that disturbance of the protein fold and structural characteristics, locally as well as globally, be kept to a minimum; otherwise the loss of activity, for instance, would be a result of conformational changes and the exchanged residue be improperly identified as catalytic. Residue substitutions, where the latter situation does not occur, can be considered as "safe".

Natural evolution has "engineered" protein structures by modifying certain molecular properties such as substrate specificity or surface charges and yet conserved the global protein topology. By comparing known conserved three-dimensional protein structures it is possible to glean hints about how this process was performed (Lesk & Chothia, 1980, 1982; Chothia & Lesk, 1986; Bashford *et al.*, 1987); rules obtained in this way are useful for designing site-directed mutagenesis experiments. Protein engineering in the laboratory often faces similar trials. For example, suppose that charges on a protein surface are to be altered to construct a cation binding site. Which amino acids near the surface would be safer to substitute to achieve the desired charge configuration?

In this work residue exchange matrices are calculated that represent point mutational preferences as observed in homologous and known three-dimensional protein structures. Alignments of primary sequences determined from spatial superposition of the main-chain C^{α} and taken from nine molecular families allowed identification of structurally equivalent residues in each of the familial sequence sets. A search was then performed for equivalent residues that had mutated but maintained similar unmutated environments defined by these atoms in contact with the central residue pairs. Such point mutations as observed in known tertiary structures are likely to be, with present-day knowledge, the closest possible mimic of *in vivo* site-directed mutagenesis.

Residue exchange statistics and their significance

were determined for all the structural equivalents in the various molecular families. The preferred and avoided substitutions were elicited from three structural contexts: buried residues, amino acids exposed beyond some water-accessible surface area threshold, and then all cases regardless of accessible state. These exchange matrices should provide considerable aid in the difficult process of deciding which residue to exchange and then with which amino acid it should be substituted to maintain protein structural integrity. The preferred exchanges are also discussed in terms of residue physicochemical characteristics.

2. Data and Methods

(a) *Aligned structures*

Aligned sequence sets were taken from 9 molecular families: globins, immunoglobulins, cytochromes *c*, serine proteases, subtilisins, calcium binding proteins, acid proteases, toxins, and virus capsid proteins. The total number of sequences, each with known 3-dimensional structure as contained in the 1989 Brookhaven database collection (Bernstein *et al.*, 1977), was 55. Table 1 lists their database code identification, protein name, species, reference for the 3-dimensional structure, and, where present, reference in which the alignment of the familial sequences used here was determined. The alignments were generally achieved by careful examination of the X-ray crystallographic structures coupled with spatial superposition of the main-chain C^{α} atoms (Rossmann & Argos, 1981). In 3 cases (calcium binding proteins, acid proteases and toxins) structures were superimposed by the present authors using the technique of Rossmann & Argos (Rossmann & Argos, 1976, 1977; Argos & Rossmann, 1979). Due to the increasing number of solved protein structures, many of those used in the present work extracted from the 1989 release of the Brookhaven database were not included in the references showing the familial alignments. These further sequences, indicated by an asterisk in Table 1, were aligned by the authors to the closest family member in both sequence and structure.

When considering statistics for buried residues (solvent-accessible surface area below an upper limit), both constant and variable domains were utilized from the immunoglobulins. However, the variable regions were excluded from the exchange matrix statistics involving surface-exposed amino acids, since large segments of the variable domain loops bind antigens and therefore are subject to special constraints. For a similar reason, side-chains contributing to subunit interface or cofactor contacts were not included in the substitution calculations.

(b) *Similarity of environment*

In a previous paper, Bordo & Argos (1990) carefully defined a measure of similarity (see S'' as given by them in eqns (1) and (3)) between 2 atomic environments surrounding structurally equivalent residues. The same measure is used here. An environment or neighborhood for a residue (called a central residue) is defined by the number of atoms and amino acid types that are within 4·5 Å (1 Å = 0·1 nm) of any side-chain atom in the surrounded residue. The similarity score S is expressed as a fraction and is defined as:

$$S = \frac{\sum_i (\bar{b}_i + \bar{s}_i \delta_i)}{\sum_i (\bar{b}_i + \bar{s}_i)}. \quad (1)$$

The denominator is simply the mean number of atoms belonging to residues present in at least 1 of the 2 environments ($\bar{b}_i$ = main-chain atoms, $\bar{s}_i$ = side-chain atoms). The mean refers to the 2 sets of atoms in each of the 2 environments. The numerator is the sum of the mean number of all main-chain atoms by the 2 environments regardless of the mutational state of the equivalent neighborhood residues plus the mean number of side-chain atoms $\bar{s}_i$ from residues that touch at least 1 atom of the mutated central residues (i.e. within 4·5 Å). The term δ_i is 0 if the ith residue is mutated and 1 if identically conserved. $\sum_i$ is over all residues that touch at least 1 of the central residues. Therefore, similarity of 2 environments will be diminished only if there are mutations in the equivalent environmental residues. That is, if structurally equivalent residues forming the neighborhood of a central residue in 1 protein structure are conserved in the other structure despite their absence in the neighborhood of the equivalent central residue in the latter structure, the similarity score is not decreased. This allows for cases where contacts made by the substituted central residue with its neighbors change only in consequence of its change in size and shape. For instance, environmental residues can move considerably to accommodate a small residue changing to a large one. Though the side-chains in contact with the larger residue are not in contact with the small one, they are nonetheless available without mutation to make contact as necessitated by the substituted residue. Water-accessible surfaces of the combined main-chain and side-chain for each residue was calculated by the procedure of Kabsch & Sander (1983).

(c) *Statistical significance of exchanges*

Counts were made for every observable substitution of central residues with similar neighborhood at a preset similarity threshold. To give statistical significance to these figures, a comparison between observed and expected number of substitutions was performed under the following hypothesis. Consider a pool of N amino acids. $N = \sum_i n_i$ ($i = 1$ to 20), where the ith amino acid type appears n_i times. The exchange $i \rightarrow j$ is a directed replacement of the amino acid i with the amino acid j (e.g. Ala → Asp) and substitution i–j refers to either $i \rightarrow j$ or $j \rightarrow i$ (e.g. Ala → Asp or Asp → Ala). There are $N(N-1)$ possible exchanges in the pool, of which $\sum_i n_i(n_i-1)$ are between residues of the same kind. Therefore, $N' = N(N-1) - \sum_i n_i(n_i-1)$ is the number of possible exchanges involving pairs of different residues. Since the observed mutations refer to only substituted residues, N', and not N, represents the pool of available exchanges. The probability $p_{i\rightarrow j}$ is then given by $n_i n_j/N'$, and the probability to observe a substitution p_{i-j} becomes:

$$p_{i-j} = 2n_i n_j/N'. \quad (2)$$

Given a total number of X observed substitutions, the expected number of substitutions n_{i-j} is therefore Xp_{i-j}.

The population n_i ($i = 1$ to 20) was calculated in the following manner. Given a set of structurally aligned sequences for a particular molecular family, each alignment column would generally contain several amino acid types. The count for the population n_i ($i = 1$ to 20) was

Table 1
Tertiary structures used in this work

Family	BRK†	Protein	Origin	Structure reference	Alignment reference‡
Hemoglobin					Lesk & Chothia (1980)
	4HHB	Hemoglobin	Human	Fermi *et al.* (1984)	
	2MHB	Hemoglobin	Equine	Ladner *et al.* (1977)	
	1FDH	Gamma globin	Human	Frier & Perutz (1977)	*
	1MBD	Myoglobin	Whale	Phillips (1980)	
	1MBS	Myoglobin	Seal	Scouloudi & Backer (1978)	*
	2LHB	Hemoglobin V	Sea lamprey	Hendrickson *et al.* (1973)	
	1ECA	Erythrocruorin	*Chironomous*	Steigemann & Weber (1979)	
	2LH1	Leghemoglobin	Lupin	Vainshtein *et al.* (1977)	
Immunoglobulins					Amzel & Poljak (1979)
	1FB4	FAB Kol	Human	Marquart *et al.* (1980)	*
	1FBJ	FAB IgA	Mouse	Navia *et al.* (1979)	*
	1FC1	FcIggI	Human	Deisenhofer (1981)	*
	1FC2	Fc	Human	Deisenhofer (1981)	*
	1IG2	Fc Kol	Human	Marquart *et al.* (1980)	*
	1MCP	FAB	Mouse	Segal *et al.* (1974)	*
	1PFC	Fc IggI	Porcine	Bryant *et al.* (1985)	*
	1REI	FAB Bence-Jones	Human	Epp *et al.* (1975)	*
	2RHE	FAB Bence-Jones	Human	Furey *et al.* (1983)	*
	3FAB	FAB New	Human	Saul *et al.* (1978)	
	2HFL	FAB IggI	Mouse	Sheriff *et al.* (1987)	*
	1F19	FAB	Mouse	Lascombe *et al.* (1989)	*
Cytochromes c					Dickerson (1980)
	155C	Cytochrome c550	*Paracoccus* D	Timkovich & Dickerson (1976)	
	3C2C	Cytochrome c2	*Rhodospirillum* R	Salemme *et al.* (1973)	
	4CYT	Cytochrome c	Bonito fish	Takano & Dickerson (1980)	
	1CYC	Ferrocytochrome c	Tuna fish	Tanaka *et al.* (1975)	*
	1CCR	Cytochrome c	Rice	Ochi *et al.* (1983)	*
	451C	Cytochrome c551	*Pseudomonas* A	Matsuura *et al.* (1982)	
Serine proteases					Craik *et al.* (1983)
	2SGA	Proteinase A	*Streptomyces* G	Moult *et al.* (1985)	
	3SGB	Proteinase B	*Streptomyces* G	Read *et al.* (1983)	
	2ALP	Alpha-lytic protease	*Lysobacter* E.	Fujinaga *et al.* (1985)	
	4CHA	Alpha chymotrypsin	Bovine	Tsukada & Blow (1985)	
	3PTB	Beta trypsin	Bovine	Marquart *et al.* (1983)	
	2TRM	Trypsin	Rat	Sprang *et al.* (1987)	*
	1TON	Tonin	Rat	Fujinaga & James (1987)	*
	2KAI	Kallikrein	Porcine	Bode *et al.* (1983)	*
	1SGT	Trypsin	*Streptomyces* G	Read & James (1988)	*
	3EST	Elastase	Porcine	Meyer *et al.* (1988)	*
	3RP2	Mast cell protease	Rat	Remington *et al.* (1988)	*
Subtilisins					Froemmel & Sander (1989)
	1SBT	Subtilisin	*B. amyloliquefacensis*	Alden *et al.* (1971)	
	2PRK	Proteinase K	Fungus	Paahler *et al.* (1984)	
	1CSE	Subtilisin Karlsberg	*B. subtilis*	Bode *et al.* (1987)	
Calcium binding proteins					
	3CLN	Calmodulin	Rat	Babu *et al.* (1988)	*
	3CPV	Ca-binding parvalbumin B	Carp	Moews & Kretsinger (1975)	*
	3ICB	Ca-binding protein	Bovine	Szebenyi & Moffat (1986)	*
	4TNC	Troponin C	Chicken	Satyshur *et al.* (1988)	*
Acid proteases					
	2APP	Penicillopepsin	Fungus	James & Sielecki (1983)	*
	2APR	Rhizopuspepsin	Mold	Suguna *et al.* (1987)	*
	4APE	Endothiapepsin	Fungus	Pearl & Blundell (1984)	*
Toxins					
	1CTX	Alpha cobratoxin	Cobra	Walkinshaw *et al.* (1980)	*
	1NXB	Neurotoxin B	Sea snake	Tsernoglou *et al.* (1978)	*
	2ABX	Alpha bugarotoxin	Krait	Love & Stroud (1986)	*
Viruses					
	2TBV	Tomato bushy stunt	Virus	Hopper *et al.* (1984)	Rossmann *et al.* (1983)
	4SBV	Southern bean mosaic	Virus	Silva & Rossmann (1985)	
	2STV	Satellite tobacco necr.	Virus	Jones & Liljus (1984)	
	1MEV	Mengo	Virus	Luo *et al.* (1987)	Luo *et al.* (1987)
	4RHV	Rhino	Virus	Arnold & Rossmann (1988)	Luo *et al.* (1987)

† The column labeled BRK gives the Brookhaven database entry name (Bernstein *et al.*, 1977).
‡ References showing structural sequence alignments used in this work. An asterisk refers to the cases where the structural alignment was performed by the authors.

Table 2
Residue counts for the nine structural protein families

Residue type	Buried†	Exposed‡	All§
Gly	161	226	445
Ala	182	250	515
Ser	108	375	533
Pro	34	194	249
Asp	28	255	315
Cys	38	23	71
Asn	33	258	313
Thr	79	341	477
Glu	11	239	255
Val	206	166	415
Gln	26	201	248
His	20	69	105
Met	49	47	107
Leu	165	135	331
Ile	125	104	265
Lys	5	297	320
Arg	9	162	193
Phe	89	88	208
Tyr	38	128	191
Trp	30	33	68

† Residues having solvent-accessible surface less than or equal to 10 Å^2. Counts are performed as described in Data and Methods.

‡ Residues having solvent-accessible surface more than or equal to 30 Å^2. Counts are performed as described in Data and Methods.

§ All residues are counted, regardless of their exposure to solvent.

increased by 1 only once for each amino acid type in the alignment column, regardless of its number of appearances. This was consistent with the counts for redundant central residue pairs. For instance, suppose an alignment position contained 3 Ala and 2 Gly residues in a particular topologic family, a total of 6 residue substitutions can be counted; however, since they are all structurally equivalent, only 1 should be taken; namely, that Gly–Ala substitution with the highest environmental similarity score. This selection is consistent with the aim of this study to find conserved neighborhoods tolerating mutant central residues. Total counts n_i ($i = 1$ to 20) were determined for all the alignment positions in all the molecular families under 3 water-accessible conditions and are given in Table 2. The probability to observe α substitutions i–j out of X trials taken from a pool of N residues ($N = \sum_i n_i$) assuming a binomial distribution is given by:

$$P_{i\text{-}j}(X, \alpha) = \binom{X}{\alpha} p_{i\text{-}j}^{\alpha}(1 - p_{i\text{-}j})^{X-\alpha}, \qquad (3)$$

where $p_{i\text{-}j}$ is given in eqn (2), and:

$$\binom{X}{\alpha} = \frac{X!}{\alpha!\,(X-\alpha)!}.$$

Given the number of observed substitutions $n_{i\text{-}j}$, it is straightforward to calculate its chance probability with eqn (3) (see e.g. Korn & Korn, 1968). If the sum of all probabilities $p_{i\text{-}j}(X, \alpha)$ for $n_{i\text{-}j} \leq \alpha \leq X$ is less than or equal to 0·05, the preference of the substitutions can be considered significant at the 95% confidence level or better. Consider the following hypothetical illustration. Suppose the pool of residues consisted of 10 amino acids for each of the 20 types ($n_i = 10$, $i = 1$ to 20), then $N = 200$ and the number of possible non-identical amino acid exchanges N' is:

$$(200 \times 199) - \sum_i (10 \times 9) = 38{,}000.$$

If, for instance, 1000 substitutions are observed ($X = 1000$), the expected $n_{i\text{-}j}$ using eqn (2), is $2 \times 1000 \times 10 \times 10/38{,}000 \sim 6$. Assume that for a given pair i–j (e.g. Ala–Thr) the observed number of substitutions $n_{\text{Ala-Thr}}$ is 12, then if

$$P_{\text{Ala-Thr}}(1000,12) + P_{\text{Ala-Thr}}(1000,13) + \ldots + P_{\text{Ala-Thr}}(1000,1000) <= 0{\cdot}05$$

the substitution preference between Ala and Thr can be considered significant with at least 95% confidence.

Table 3
Number of substitutions for buried residues involving volume and polarity alterations

Similarity (%)†	100	95	90	85	80
Observed substitutions	12	34	65	124	206
Total number with volume change > 1 methyl group	—	1	9	24	57
Total number with polarity group change	2	2	14	33	63
Hydrophobic/hydrophilic substitutions	—	—	1	1	1

† Percentage similarity threshold of central residue environments (see eqn (1)).

3. Results and Discussion

Table 2 lists the residue population for each of the amino acids in the three structural states examined for central residue substitutions: (1) buried in the protein core (solvent-accessible surface for both residues ≤ 10 Å^2); (2) exposed (solvent-accessible surface area ≥ 30 Å^2); and (3) all the possible accessibility states allowed. The residue pool represents, under the constraints discussed in Data and Methods, the composition of amino acids available for possible substitutions. These populations are important in calculating the substitution statistical significance (see Data and Methods).

In a previous paper (Bordo & Argos, 1990), substitution statistics were gathered from only one sequence family (globins) and for only buried residues. The buried exchange counts given here increased by at least a factor of 5 from the addition of eight sequence families (Table 1). The basic trends observed were nonetheless conserved. The results in Table 3 make this salient. Very few of the total substitutions show volume changes greater than one methyl group (~ 35 Å^3) and a movement (referred to as a "jump") to another polarity group (Grantham, 1974) where the three possible groups are defined (1 letter code used) by (WYFMCILV), (PATGS) and (HKRQDEN). These constraints imply considerable impact on the development of protein cores in structures maintaining main-chain fold; a detailed discussion can be found in the earlier work (Bordo & Argos, 1990). All ensuing work given here is unique to this report.

Table 4
Number of substitutions for exposed residues involving volume and polarity alterations

Similarity (%)†	100	95	90	85	80
Observed substitutions	100	152	322	560	941
Total number with volume change > 1 methyl group	28	54	124	268	466
Total number with polarity group change	42	69	153	280	547
Hydrophobic/hydrophilic substitutions	3	5	19	39	78

† Percentage similarity threshold of central residue environments (see eqn (1)).

Table 4 lists similar statistics (volume and polarity group alteration counts) for exposed residues with similar environments. It is clear that they display considerable point mutation freedom compared to the buried residues. Approximately one-third to one-half of the substitutions (depending on the percentage similarity of the neighborhood) involve changes in polarity group or volume alterations greater than one methyl group, whereas only about 15% of the buried substitutions involved such changes. However, few side-chains (~5%) alter the sign of their charge or jump (~3%) between opposite polarity groups (i.e. hydrophobic–hydrophilic) despite their exposure.

It was insisted that each of the two substituted residues have a water-accessible surface area of at least 30 Å^2 to be deemed exposed. This represents approximately a hole just large enough for a methyl group to pass through and was found from the previous globin statistics (Bordo & Argos, 1990) as well as the present data (not shown) to be the minimal exposure at which radical volume and polar alterations between exchanged central residues are observed.

Figure 1 shows the actual exchange counts for (a) buried, (b) exposed and (c) all cases where the central residue environments were 90% or greater (lower matrix half) and 70% or greater (upper matrix half) in similarity. The symbols plus (preferred exchange) and minus (avoided exchange) are shown in the upper half of matrices if the counts were reliable at the 95% confidence level or better as well as consistently preferred or shunned for at least two similarity levels within a range of 100% to 70% calculated in steps of 5%. As expected, the 70% similarity data produced the most observed exchange counts and the greatest number of substitutions deemed significant. However, given the lessened neighborhood similarity, noise is increasingly introduced; nonetheless, trends are preserved from the 90% to 70% levels (Fig. 1).

Several interesting substitution trends are observable in the Figure 1 exchange matrices. Though the high count substitutions are not always deemed statistically significant, they represent a useful starting point in deciding which substitutions to try in structure-altering experiments as site-directed mutagenesis or protein engineering. It will take considerable time and effort to produce sufficient X-ray crystallographic protein structures to determine the significance of all the possible substitutions.

For the protein core, residues within each of the following subsets are generally interchangeable with high statistical significance: (A, G), (A, V), (N, D), (M, L), (F, L), (F, Y), (A, S, T), (V, I, L) and (Y, W). This is shown diagrammatically in Figure 2. In an examination of the counts alone, surprising results can be found for many of the amino acid types. While Thr can exchange with Ala and Ser, Asn is the next most desirable. Cys prefers Ala or Val as substitutes. Though Val can rather freely go to Ala, Ile and Leu, Ile prefers primarily only Val and Leu. Met and Phe favor Leu, rather than Ile, as an ersatz. For exposed substitutions unexpected results are also in evidence. Gly prefers Asn as the most desirable charged or polar substitute. If Ala must be replaced by a charged residue, Lys and Glu are statistically favored. Ser prefers Asp and Asn and not Glu, Lys or Arg, while Thr is the most favored substitute. Asp especially avoids Tyr at the surface. Val's favorite partners are Ile and Leu, while Tyr prefers Phe. Interestingly, the hydrophobic residues Val, Leu and Ile tend to substitute amongst themselves despite some exposure at the surface. If an exposed Val must be changed to a charged residue, Lys is the best candidate; and so forth.

Some substitutions are consistently allowed regardless of exposure or buriedness (Fig. 2). Among the highly significant preferred exchanges, in single letter code, are (C, A), (S, A), (T, A), (N, D), (T, S), (V, I, L) and (F, Y).

Calculating the logarithm of the ratio of the observed to expected counts for each possible substitution and for all observed cases having 70% environmental similarity (Fig. 1(c), upper right matrix), it was possible to build a scoring matrix analogous to that determined by Dayhoff *et al.* (1978). The correlation coefficient between the elements of the two matrices was 0·64. It would not be expected that the two matrices correlate well as the results of this work concern single substitutions over only close molecular generations, while the Dayhoff *et al.* observations are cumulative over many and multiple mutations.

The matrices listing preferred or safe and avoided or unsafe substitutions taken from actual tertiary structures should prove exceedingly useful in site-directed mutagenesis and protein engineering experiments. It would be helpful to ascertain if a residue is exposed or buried before choosing a substitution. If the protein three-dimensional structure is known, this information is evident. If only the sequence has been determined, secondary structure prediction and/or a hydrophobicity plot (for a review, see Argos, 1990) should provide a good guess as to the appropriate solvent-accessible state of the residue in question. If not, the exchange counts taken from all residues in the familial sequence sets are given in Figure 1(c).

	G	A	S	C	T	P	D	V	N	L	I	Q	M	E	H	K	F	R	Y	W
G		23+	6	3	5	0	0	8-	0	4	3	1	1	0	0	0	1-	0	0	0
A	9		16+	4	13+	3	1	32+	3	14	8	3	2	1	0	0	5	1	2	0
S	1	5		1	16+	1	2	5	8	4	3	0	1	0	0	0	3	0	1	1
C	0	1	0		0	0	0	4	0	0	1	0	0	0	0	0	1	0	0	0
T	0	4	5	0		1	1	10	2	3	6	0	4	1	0	0	2	0	0	0
P	0	0	0	0	0		0	1	0	0	0	0	0	0	0	0	1	0	0	0
D	0	0	0	0	0	0		0	3+	0	0	0	0	0	0	0	0	0	0	0
V	1	2	1	1	1	0	0		0	34+	39+	0	11	0	3	0	6	0	3	0
N	0	0	1	0	1	0	1	0		1	0	2	1	0	0	0	1	0	1	0
L	0	1	0	0	0	0	0	3	0		19+	2	15+	0	1	0	13+	0	4	5
I	0	1	0	0	1	0	0	10	0	3		2	4	0	0	0	4	1	1	4
Q	0	0	0	0	0	0	0	0	0	0	0		1	1	0	0	1	0	1	1
M	0	0	0	0	1	0	0	1	1	2	1	0		1	0	0	3	0	1	1
E	0	0	0	0	0	0	0	0	0	0	0	1	0		0	0	0	0	0	0
H	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	1	1	0	0
K	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	1	0	0
F	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0		0	6+	1
R	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0		1	0
Y	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0		4+
W	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	

(a)

	G	A	S	C	T	P	D	V	N	L	I	Q	M	E	H	K	F	R	Y	W
G		29+	33+	1	20	19	23	3	30+	1-	1-	7	1	14	4	23	1	7	5	1
A	8		53+	2	30+	19+	23	6	26	5	5	20	2	32+	7	34+	3	9	4-	2
S	10	17		1	80+	29	45	13	50	6-	4	34	2	25	14	35	2-	20	4-	0
C	0	0	0		2	1	0	0	0	2	1	0	0	1	0	0	0	0	0	0
T	2	9	21	0		20	20	17	34	14	10	30	5	25	7	38+	7	17	7-	2
P	3	5	6	0	3		21	6-	12	3-	2	11	1	19	8	21	0	6	1	1
D	2	2	9	0	1	5		7-	45+	6	4	17	1	42+	4	25-	2	5	2-	0
V	1	2	2	0	3	0	0		6	14+	14+	7	1	10	0	15	2	8	4	0
N	7	3	7	0	3	0	11	1		10	5	18	1	13	6	32	5	7	6-	2
L	0	1	1	0	1	0	1	2	2		9+	13	4	8	1	14	3	7	7	3
I	0	1	1	0	2	0	0	2	0	4		6	3	7	1	7	6	2	3	1
Q	0	3	4	0	6	1	4	1	3	1	0		5	23+	6	29+	1	11	3	0
M	0	0	1	0	0	0	0	0	0	1	1	1		1	1	3	2	1	0	0
E	3	6	5	0	4	4	10	1	2	1	1	3	0		3	30	3	6	5	0
H	2	1	0	0	1	1	0	0	1	0	0	2	0	0		9	2	1	4	1
K	4	10	2	0	12	2	2	2	6	3	0	5	0	6	0		4	28+	3-	3
F	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0		1	11+	3
R	1	1	3	0	1	0	1	2	1	0	0	1	0	0	1	7	0		3	2
Y	0	1	0	0	0	0	0	1	0	2	1	0	0	0	0	1	3	0		4
W	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	

(b)

	G	A	S	C	T	P	D	V	N	L	I	Q	M	E	H	K	F	R	Y	W
G		79+	65	4	45	25	34	16-	44+	9-	7-	19	6	23	8	31	4-	13	10-	3
A	23		110+	8	73+	35	33	63+	39	30-	24	39	11	46+	12	54	10-	21	14	4
S	14	35		5	128+	40	59+	31-	74+	17-	16	46	6-	32	16	53	8-	31	11-	4
C	0	0	0		7	4	0	6	0	3	3	1	0	2	0	0	2	0	0	1
T	7	21	43	0		31	29	48	47+	32	29	42	15	34	11	58+	15	29	10-	1
P	5	10	7	0	7		25	12-	16	7-	3-	11	1	22	8	22	3	9	4-	1
D	7	3	11	0	4	5		12-	61+	9-	9-	19	1-	47+	4	25	4-	9	2-	0
V	2	10	6	1	8	1	2		9-	65+	77+	13	19	18	7	21	19	16	12	0-
N	9	6	12	0	5	0	15	1		13-	8-	20	3	17	8	36	7-	10	9-	2
L	0	2	1	0	3	1	0	7	2		45+	21	30+	15	4	17	22	11	14	9
I	0	3	2	0	7	0	2	17	0	10		8	9	9	2-	7-	15	6-	6	5
Q	1	6	6	0	8	1	4	4	3	1	0		9	29+	10	38+	2-	18	5	2
M	0	0	1	0	4	0	0	2	1	7	3	1		3	3	6	5	2	2	1
E	4	6	6	0	8	5	12	3	2	1	2	9	0		4	31	3-	10	5-	0
H	3	2	0	0	0	2	0	0	1	0	0	0	0	1		8	5	3	8	2
K	5	11	5	0	14	2	2	2	7	2	0	8	0	6	1		4-	38+	3-	3
F	0	0	0	0	1	0	0	0	0	4	2	0	1	0	0	0		4-	23+	9+
R	2	3	4	0	3	1	2	2	2	1	0	4	0	0	2	9	0		4	2
Y	0	2	0	0	0	0	0	2	0	2	1	1	0	0	1	1	5	0		12+
W	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	1	

(c)

Figure 1. Observed substitutions for (a) buried, (b) exposed and (c) all cases. The lower halves of the matrices give substitution counts for central residues with 90% or greater similar environments, while the upper halves are for 70% or greater similarity. When counts show a statistically meaningful (95% or greater confidence) increase or decrease compared to the expected figures for at least 2 similarity levels ranging from 100% to 70% in steps of 5%, with the trend being consistent, a + or − sign is given to indicate preferred or avoided substitutions, respectively. In the exposed data, immunoglobulin variable domains were not included.

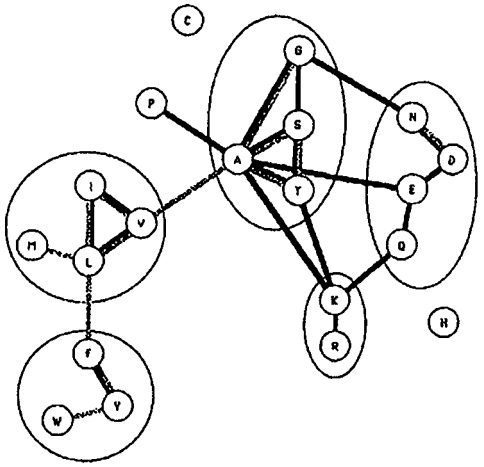

Figure 2. Statistically preferred (95% or greater confidence level as indicated by a + in Fig. 1) substitutions observed in buried residues (grey segments) and exposed residues (black segments) are shown. Residues roughly equivalent are grouped together in 5 subsets, which generally correlate with side-chain physicochemical properties.

Lim & Sauer (1989) have performed mutation experiments on λ repressor protein core side-chains and the mutants were assayed for functionality and stability. Interestingly, all of the single protein core mutants could have been predicted from this work (Bordo & Argos, 1990).

Site-directed mutagenesis is an important tool in probing the structural and functional significance of particular residues within a protein sequence (for reviews, see Knowles, 1987; Shaw, 1987). Amino acid residues might be altered to check for their participation in catalysis, cofactor or substrate binding, molecular and receptor recognition, domain interfaces, oligomeric interactions, and the like. It is essential in such experiments that the protein fold, locally and globally, not be perturbed; otherwise, loss of activity or whatever aspect is under study would be incorrectly ascribed to the mutated residue. "Safe" substitutions are thus requisite for the success of the mutant probe as an indicator of critical residues in structure and function. This work provides exchange matrices that should be directly applicable in maintaining the fold and that are taken from known three-dimensional protein structures with diverse folds. Of course, the results represent general trends and cannot be expected to work in every local context, but they should be a great improvement over randomly selected substitutions and act as a good guide regarding what to substitute and what not to substitute. For example, suppose Cys were a suspected active site residue. If exposed or buried, though the substitution data base is not sufficient to identify statistically significant exchanges for Cys, the observed substitutions counts would recommend Ala; if the Cys is likely to be buried, Val is also a possible candidate.

Zvelebil & Sternberg (1988) examined several known tertiary structures and determined that His is the most frequently occurring catalytic residue. Assuming its exposure to the solvent, the exchange matrix suggests Ser as the safest substitution. In the review by Shaw (1987) on specific point mutations for several molecular species, the Gly–Ala substitution is one of the most frequent mentioned. Apparently the proteins maintained their fold while proven assays displayed altered activity. The exchange matrices presented in this work suggest the Gly–Ala substitution as highly significant in the buried or exposed states.

In protein engineering as well as molecular modeling, where new structures are built from those with known tertiary and homologous primary structures (for a review, see Sali *et al.*, 1990), it is often crucial to know which residues can be substituted safely. Can a substituted residue in a molecular model be placed in the same environment displayed by the known native structure? For instance, if a His is to be introduced in an exposed loop to engineer cation binding, would it be safer to substitute a Ser, Glu, Asn or Lys in the known structure? The exchange matrices of Figure 1 provide direct answers. In fact, Sali *et al.* (1990) in their review on modeling cite only two specific examples where residues are allowed limited choices due to folding requirements. Both involve constrained Ser–Thr substitutions in buried β-strands where the side-chain oxygen atoms bond to main-chain atoms. Among the preferred exchanges, the Ser–Thr one is highly preferred both in the exposed and buried substitutions matrices reported here (Fig. 2). A further protein engineering example would involve a desired residue substitution to stabilize a predicted or known helix. The exchange should be from a residue of lower to higher helical preference (Palau *et al.*, 1982). Combining this requirement with the exchange matrix counts of Figure 1 should provide a very rational substitution, especially if the tertiary structure is not known, which is typically the situation. For example, if Ile were buried and part of a helix is to be stabilized, the matrix of Figure 1(a) suggests Leu and then Met as likely substitution candidates.

Malcolm *et al.* (1990) have published results of mutants of game bird lysozymes. Point mutations on *in vivo* triplets Thr40-Ile55-Ser91 (TIS) or Ser40-Val55-Thr91 (SVT) included, respectively, TVS, SIS, TIT and SVS, SIT, TVT. The mutants were assayed for thermal stability and it was found that TIT, SIT and TVT were more stable than the respective wild-type and TVS, SIS and SVS less so. The buried-residue exchange matrices in this work would predict that Val → Ile and Ser → Thr would be ideal substitutions to preserve main-chain fold and enhance thermal stability under the assumption that increasing the volume of a side-chain within one methyl group would result in better hydrophobic packing to maintain the protein structure. In every case, this is exactly what occurred experimentally. In fact, when the exchange from the wild-

type involved a volume decrease, the fold was maintained but thermal stability diminished.

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Minimal BH3 Peptides Promote Cell Death by Antagonizing Anti-apoptotic Proteins*

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The pro-apoptotic "BH3 domain-only" proteins of the Bcl-2 family (*e.g.* Bid and Bad) transduce multiple death signals to the mitochondrion. They interact with the anti-apoptotic Bcl-2 family members and induce apoptosis by a mechanism that requires the presence of at least one of the multidomain pro-apoptotic proteins Bax or Bak. Although the BH3 domain of Bid can promote the pro-apoptotic assembly and function of Bax/Bak by itself, other BH3 domains do not function as such. The latter point raises the question of whether, and how, these BH3 domains induce apoptosis. We show here that a peptide comprising the minimal BH3 domain from Bax induces apoptosis but is unable to stimulate the apoptotic activity of microinjected recombinant Bax. This relies on the inability of the peptide to directly induce Bax translocation to mitochondria or a change in its conformation. This peptide nevertheless interferes with Bax/Bcl-xL interactions *in vitro* and stimulates the apoptotic activity of Bax when combined with Bcl-xL. Similarly, a peptide derived from the BH3 domain of Bad stimulates Bax activity only in the presence of Bcl-xL. Thus, BH3 domains do not necessarily activate multidomain pro-apoptotic proteins directly but promote apoptosis by releasing active multidomain pro-apoptotic proteins from their anti-apoptotic counterparts.

Apoptosis is a highly regulated process of cell demise triggered by internal or external stimuli. Most apoptotic signaling pathways converge on the mitochondrion and lead to a change in the mitochondrial outer membrane permeability (1). As a result, diverse apoptogenic proteins, such as cytochrome *c* (which allows the activation of a caspase 9/caspase-3 cascade via the cytosolic adapter Apaf-1), Smac/DIABLO, "apoptosis-inducing factor," endonuclease G, and HtrA2, are released from this organelle. The response of the mitochondrion to upstream stimuli is a critical control point in the regulation of apoptosis. It is crucial, therefore, to understand how this organelle integrates a great variety of death signals.

Bcl-2 family members are major regulators of mitochondrial integrity (2). Anti-apoptotic members such as Bcl-2 and Bcl-xL display sequence conservation throughout all four Bcl-2 homology domains (BH1–4), whereas the pro-apoptotic Bax and Bak possess homology in BH1–3 domains. These multidomain pro-apoptotic proteins have the innate ability to alter mitochondrial integrity, possibly via their ability to induce channels in the mitochondrial membrane (3). The ratio between anti-apoptotic and multidomain pro-apoptotic Bcl-2 family members helps determine the cellular susceptibility to death stimulation (4). NMR structural analysis of the Bcl-xL-BakBH3 peptide complex has revealed that the BH3 domain of Bak binds to a hydrophobic cleft formed by the BH1, BH2, and BH3 domains of Bcl-xL (5). Anti-apoptotic and multidomain pro-apoptotic Bcl-2 family members may thus engage, by a BH3 domain-dependent mechanism, in the formation of heterodimers in which they mutually antagonize each other's function (6).

The Bcl-2 family includes a third subgroup of pro-apoptotic members that display sequence homology only with the BH3 domain (7). These BH3 only proteins seem to act as the afferent effectors of various pro-apoptotic and anti-apoptotic signals (8). For instance, both Noxa and Puma are transcriptionally regulated by p53 (9–11); activation of Bid by caspase 8-mediated cleavage recruits the mitochondrial apoptotic pathway into death receptor signaling (12, 13), whereas the activity of Bad is negatively regulated by the Akt/protein kinase B survival signaling pathway (14). Murine cells lacking both multidomain Bax and Bak display long term resistance to mitochondrial damage and cell death induced by all BH3-only proteins tested (15–17). Thus, in mammals, activation of BH3 only proteins integrate diverse apoptotic stimuli into one single pathway by triggering Bax/Bak-mediated mitochondrial dysfunction.

All BH3-only proteins have the ability to bind to and to functionally antagonize anti-apoptotic Bcl-2 family members (8). The BH3 domain-dependent mechanism by which Bax/Bak pro-apoptotic function is recruited remains poorly characterized. Indeed, multidomain proteins, in viable cells, reside as inactive proteins located either at the mitochondria (Bak) or in the cytosol (Bax) (18). The direct binding of Bid to either Bax or Bak is sufficient to activate those proteins and allow their pro-apoptotic assembly in the mitochondrial membrane (19–21). Very recent evidence (22, 23) using synthetic peptides has indicated that the BidBH3 domain itself functions as a specific death ligand. These studies nevertheless revealed evidence for another functional subset of BH3 domains, which lack the ability to activate directly Bax/Bak but retain the ability to bind to anti-apoptotic Bcl-2 family members (22). It has remained unclear whether such BH3 domains can induce apoptosis and, if so, by which mechanism.

To explore this question further, we have analyzed in this study the apoptotic effects of a minimal BH3 domain synthetic peptide, comprising the critical 16 residues of the defined Bax

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BH3 domain (BaxBH3 (57–72)). These 16 residues contain sufficient information to bind to (5), and functionally antagonize (24), Bcl-xL and to induce specifically Bax/Bak-mediated apoptosis (24). We show here that, despite these properties, BaxBH3 cannot efficiently induce Bax translocation to mitochondria, evoke a change in Bax conformation, nor stimulate the apoptotic activity of recombinant Bax, whereas it can stimulate that of a mutant form of Bax that exhibits an increased ability to homodimerize. BaxBH3 interferes with Bax/Bcl-xL interactions in cell-free experiments and stimulates the apoptotic activity of Bax combined with Bcl-xL. Similarly, a peptide derived from the BH3 domain of Bad was also unable to stimulate Bax activity by itself but cooperated with Bcl-xL to activate it. Taken together, these data support a model in which BH3 peptides that mainly act as antagonists of anti-apoptotic Bcl-2 family members can induce apoptosis by interfering with the interaction between multidomain pro- and anti-apoptotic proteins.

MATERIALS AND METHODS

Reagents and Antibodies—The following antibodies were used: monoclonal anti-cytochrome *c* antibody 6H2B4 from Pharmingen; polyclonal anti-Bax BaxNT antibody from Upstate Biotechnology, Inc.; and monoclonal anti-Bax 2D2 and 6A7 antibodies from R & D Systems. The polyclonal anti-Bax TL41 antibody was raised against the BH3 domain of Bax (residues 57–72) (24). Horseradish peroxidase-conjugated antibodies and enhanced chemiluminescence reagents were obtained from Amersham Biosciences. Fluorescent Alexa 568™-conjugated secondary antibodies were obtained from Molecular Probes. Unless indicated, all other reagents used in this study were obtained from Sigma.

Peptides and Recombinant Proteins—The high pressure liquid chromatography-purified BaxBH3 (KKLSECLKRIGDELDS), BaxBH3 L63A (KKLSECAKRIGDELDS), and BadBH3 (QRYGRELRRMSDEFVD) peptides were obtained from Genosys (Cambridge, UK).

Histidine-tagged human Baxα, BaxΔART, and Bcl-xL were obtained by subcloning of the coding regions into pDEST17 plasmid (Gateway, Invitrogen) as described previously (25). His-tagged proteins were expressed in the *Escherichia coli* strain XL-1 blue and purified by chromatography on nickel-Sepharose acid resin according to the manufacturer's instructions. Recombinant proteins were dialyzed against PBS[1] and stored at −80 °C until used. The purification of GST, GST-Bcl-xL, and tBid from bacterial lysates has been described previously (24, 26).

Microinjection Experiments—Rat-1 fibroblasts, grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS and 0.1 mM glutamine, were seeded on sterile coverslips the day prior to microinjection. Microinjection was performed as described previously (27) using an InjectMan NI2 micromanipulator and a FemtoJet injector from Eppendorf (Germany). Identical, standardized conditions of pressure (100 hPa) and time (0.1 s) were used in all experiments. Peptides and/or recombinant proteins were diluted in PBS together with dextran 70 kDa-conjugated lysine-fixable Oregon Green® (Molecular Probes, 0.5% final concentration) as a co-injection marker. Typically, 100 cells were microinjected for each condition in each experiment. The percentage of positive (*i.e.* fluorescent) cells exhibiting morphological features of apoptosis following microinjection was evaluated as described previously (27) using an inverted fluorescence microscope (DMIRE2, Leica, France).

Immunocytochemistry—Immunocytochemical staining of microinjected cells was performed as described previously (24). Both the primary polyclonal anti-Bax NT antibody and the anti-rabbit secondary antibodies were used at a 1:200 dilution. Images presented in this study were collected on a Leica TCS NT confocal microscope with a 100 × 1.3 NA Fluotar objective (Leica, France).

Cell-free Assays—Mitochondria were isolated from normal rat liver as described previously (28). For mitochondrial targeting assays, [^{35}S]Met-labeled proteins (Amersham Biosciences) were synthesized from cDNAs using the TNT-coupled Transcription/Translation system (Promega, France) and quantified as described previously (28). Radiolabeled Bax (4 fmol) was incubated with isolated mitochondria (2.5 mg proteins/ml) and with the indicated concentration of recombinant tBid or BaxBH3 at 30 °C for 1 h in 40 μl of standard import buffer (250 mM sucrose, 80 mM KCl, 10 mM $MgCl_2$, 10 mM malic acid, 8 mM succinic acid, 1 mM ATP-Mg^{2+}, 20 mM MOPS, pH7.5). When indicated, ^{35}S-Bax was preincubated with unlabeled GST or GST-Bcl-xL (2 fmol) for 30 min prior to incubation with mitochondria in the presence or in the absence of BaxBH3 (200 nM). Radiolabeled proteins bound to the mitochondria were recovered by centrifugation of the incubation mixture at 8,000 × *g* for 10 min at 4 °C, separated by SDS-PAGE, and analyzed by scanning with a PhosphorImager (Amersham Biosciences) followed by quantification with IPLab gel program (Signal Analytics).

For cytochrome *c* release assays, recombinant Baxα (5 nM) was preincubated with either GST or GST-BclxL (2.5 nM) for 30 min prior to its incubation with mitochondria (1 mg of proteins/ml) in the absence or in the presence of BaxBH3 (200 nM) for an additional hour in 100 μl of import buffer. Mitochondria were then recovered, and the amount of mitochondrial cytochrome *c* was analyzed by Western blotting and quantified with IPLab gel program (Signal Analytics).

Protein Binding Experiments—In vitro His-protein binding assays were performed as described previously (25). Briefly, each radiolabeled protein (4 or 8 fmol as indicated) was incubated with an equimolar concentration of either His-tagged Bcl-xL or Baxα immobilized on nickel-Sepharose in 50 μl of binding buffer (142 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES (pH 7.4), 0.5 mM dithiothreitol, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, and a mixture of other protease inhibitors) at 4 °C for 2 h. BaxBH3 or BaxBH3 L63A (200 nM final concentration) was then added, and the mixture was incubated for an additional hour. Protein complexes were then centrifuged at 13,000 × *g* for 5 min at 4 °C, washed three times in binding buffer, eluted in elution buffer (50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, and 250 mM imidazole), separated by SDS-PAGE, and analyzed with a PhosphorImager as described above. For mild proteolysis experiments, both the pellet and the supernatant from the first centrifugation at 13,000 × *g* were treated with trypsin (1 mg/ml) for 15 min at 4 °C. Proteolysis was then stopped by the addition of 10 mg/ml soybean trypsin inhibitor prior to analysis as described above. To assess directly the sensitivity of radiolabeled Bax to trypsin treatment in the absence of His-tagged Bcl-xL, the radiolabeled protein (8 fmol) was incubated with tBid (8 fmol, 0.2 nM final concentration), the indicated peptides (200 nM), or was left untreated in 40 μl of binding buffer for 1 h at 30 °C prior to trypsin treatment as described above.

For immunoprecipitation experiments, soluble His-tagged Bcl-xL-radiolabeled Bax complexes were prepared as follows. Radiolabeled Bax (20 fmol) was incubated with 20 fmol of His tagged Bcl-xL immobilized on nickel-Sepharose in 250 μl of binding buffer at 4 °C for 2 h; protein complexes were then centrifuged at 13,000 × *g* for 5 min at 4 °C, washed three times in binding buffer, and then eluted in 20 μl of elution buffer. SDS-PAGE analysis and quantification using a PhosphorImager as described above showed that the resulting soluble complexes contained in average 4 fmol of radiolabeled Bax. These soluble complexes, or equivalent amounts of free radiolabeled Bax (4 fmol), were incubated with an equimolar amount of tBid (0.2 nM final concentration) or with the indicated peptides (200 nM) for 1 h at 30 °C in 40 μl of standard import buffer. The anti-Bax 2D2, 6A7, or TL41 antibodies were then added (1:10 final dilution) for an additional 1-h incubation at 4 °C. Antibody-protein complexes were then isolated by incubation with either Zysorbin-G (for the 6A7 and 2D2 antibodies) or Zysorbin (for the TL41 antibody) followed by centrifugation and washes according to the manufacturer's instructions (Zymed Laboratories Inc.). SDS-PAGE analysis and quantification of the immunoprecipitated and radiolabeled proteins using a PhosphorImager were performed as described above.

RESULTS

BaxBH3 Does Not Stimulate the Cytotoxic Activity of Wild Type Bax—In order to investigate the mechanism of action of BH3 domains, we used a synthetic peptide comprising the known BH3 domain of Bax (residues 57–72), BaxBH3. This peptide interacts with (5), and functionally antagonizes (24), Bcl-xL. Moreover, upon microinjection into fibroblasts, it induces apoptosis by a mechanism that requires either Bax or Bak (24). One possible reason for this is that this peptide directly activates the multidomain proteins, essentially acting as a death ligand (22). This notion appears consistent with its reported ability to interact physically with full-length Bax (29).

We reasoned that if BaxBH3 can directly activate Bax, it should be more efficient in inducing apoptosis in cells with

[1] The abbreviations used are: PBS, phosphate-buffered saline; FCS, fetal calf serum; MOPS, 4-morpholinepropanesulfonic acid; IVTR, *in vitro* translated radiolabeled; GST, glutathione *S*-transferase.

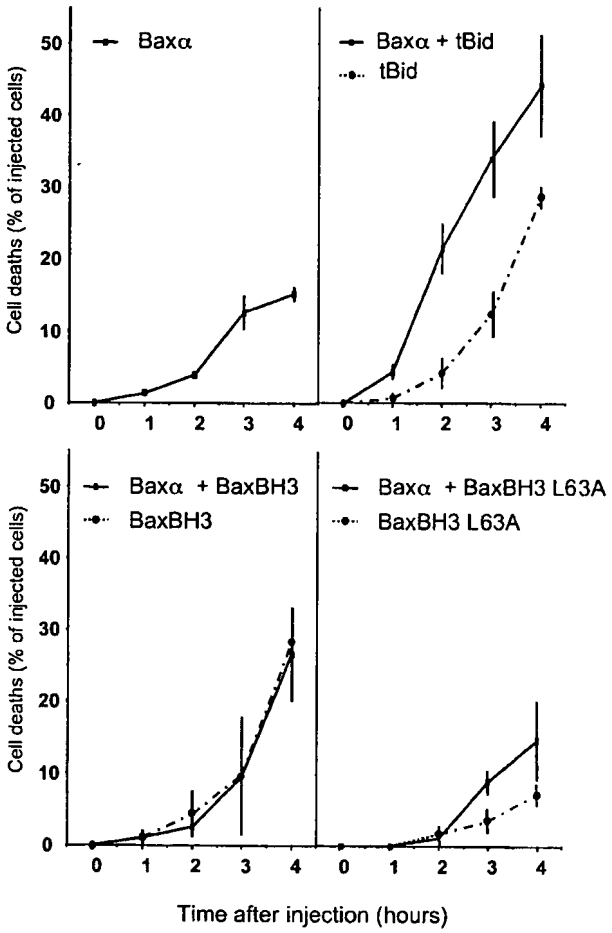


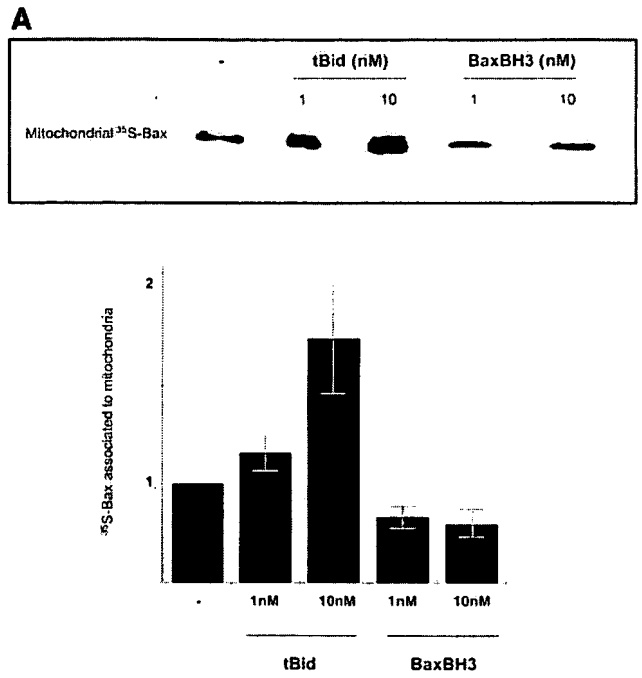
FIG. 1. **Effect of BaxBH3 on the pro-apoptotic activity of Baxα.** His-tagged recombinant Baxα (0.5 nM), tBid (0.5 nM alone or together with 0.5 nM Baxα), BaxBH3 (0.5 nM alone or together with 0.5 nM Baxα), and BaxBH3 L63A (0.5 nM alone or together with 0.5 nM Baxα) were mixed with Oregon Green dextran (0.5% w/v) in PBS and microinjected into Rat-1 fibroblasts, grown in 10% FCS. Cells were then incubated at 37 °C, and death of microinjected cells was assessed morphologically by fluorescence microscopy at the indicated times. Data are means (±S.E.) of at least three independent experiments.

increased Bax levels; on the other hand, we would expect to see no increased sensitivity to BaxBH3 in these cells if BaxBH3 were unable to stimulate Bax function directly. We therefore made use of a recombinant full-length Bax (Baxα) and assessed its effect on whole intact cells in the presence or in the absence of the BaxBH3 peptide. The recombinant protein used was prepared in the absence of detergent, in order to prevent the artifactual activation of Bax reported to occur during the purification procedure (30). This purified Baxα protein did not associate with Baxα in cell-free experiments ((25) see also Fig. 6). It is therefore unlikely to adopt a homodimeric structure. As shown in Fig. 1 (*top left panel*), microinjection of recombinant Baxα (0.5 nM) induced limited apoptosis even 4 h following injection. In order to check that this recombinant protein can be activated by certain stimuli, we co-injected Baxα with a recombinant protein equivalent to the caspase 8 cleavage product of Bid (tBid, 0.5 nM) (26). This led to far more widespread apoptosis than that observed upon microinjection of either Baxα or tBid alone (Fig. 1, *top right panel*). Apoptotic synergy between these two proteins was evident as early as 2 h after microinjection, strongly supporting the view that recombinant Baxα by itself is poorly apoptogenic but can be activated by signals such as Bid activation. Of note, the fact that subnanomolar quantities of tBid and Baxα efficiently induced apoptosis is consistent with a previous report (31) showing that similar concentrations of tBid elicit significant mitochondrial dysfunction and cell death.

We then analyzed whether BaxBH3 stimulated the pro-apoptotic function of Baxα. Microinjection of 0.5 nM BaxBH3 induced apoptosis to a certain degree (Fig. 1, *bottom left panel*), whereas microinjection of the mutant BaxBH3 L63A (0.5 nM), which does not interact with Bcl-xL, had only a limited effect on cell viability (Fig. 1, *bottom right panel*). In sharp contrast to what we observed for tBid, co-injection of Baxα and BaxBH3 did not result in an increased apoptosis in the microinjected cells (Fig. 1, *bottom left panel*). A similar lack of effect on Baxα activity was observed with the mutant BaxBH3 L63A peptide (Fig. 1, *bottom right panel*). Thus, BaxBH3 induces apoptosis, but it does not impact on the apoptotic activity of the native form of Bax.

BaxBH3 Neither Stimulates Bax Association with Mitochondria nor Significantly Modifies Its Conformation—Bax in its native conformation is only poorly targeted to mitochondria, and it acquires the ability to interact with its mitochondrial site of action only in response to certain death stimuli, including Bid activation (32). We therefore asked whether the lack of effect of BaxBH3 on Baxα apoptotic function depended upon its inability to stimulate Baxα targeting to mitochondria. For this purpose, we used an *in vitro* system employing the ^{35}S-labeled transcription-translation product of Bax cDNA in rabbit reticulocyte lysate together with purified mitochondria from rat liver. Previous analysis of Bax association with mitochondria in such a system has confirmed that Bax targeting to mitochondria is mostly inefficient but that it can be triggered by addition of cytosolic extracts from apoptotic cells (28, 33) or induced by specific mutations that serve to increase Bax apoptotic activity (25, 34). As a positive control, we checked that the addition of low concentrations (1–10 nM) of tBid stimulated the targeting of *in vitro* translated, radiolabeled (IVTR) Baxα to isolated mitochondria in a dose-dependent manner (Fig. 2*A*). In sharp contrast, the addition of BaxBH3 at 10 nM did not stimulate Baxα association to mitochondria (Fig. 2*A*). Increased concentrations of the peptide, up to 10 μM, failed to exhibit any effect on Baxα targeting (not shown, see also below in Fig. 5). We conclude that BaxBH3 is unable to stimulate Baxα association to mitochondria.

The ability of certain stimuli, such as Bid activation, to induce translocation of Bax to mitochondria has been ascribed to their ability to induce a change in Bax conformation. Such change in Bax is accompanied by the exposure of an epitope located in its N terminus, which can be detected in whole cells by immunohistochemical analysis with a specific antibody (BaxNT (35)). To understand better the inability of BaxBH3 to induce Bax translocation, we asked whether BaxBH3 or tBid, when microinjected into Rat-1 cells, induced exposure of this epitope in endogenous Bax. We used subnanomolar doses of both tBid and BaxBH3, which we had shown by themselves induce only modest apoptosis 3 h following microinjection (see Fig. 1). Microinjection was performed in the presence of the broad range caspase inhibitor benzyloxycarbonyl-VAD-fluoromethyl ketone (50 μM) to avoid caspase-induced changes in Bax conformation (32). Cells were fixed 3 h after microinjection with either tBid or BaxBH3 (0.5 nM) and immunostained using the conformation-specific BaxNT antibody as described previously (24). As shown in Fig. 2*B*, a significant portion of Rat-1 fibroblasts microinjected with tBid (0.5 nM) exhibited a marked increase in Bax NT immunostaining. This increased staining appeared as a punctate, extranuclear pattern consistent with a mitochondrial localization for the modified Bax molecules (see



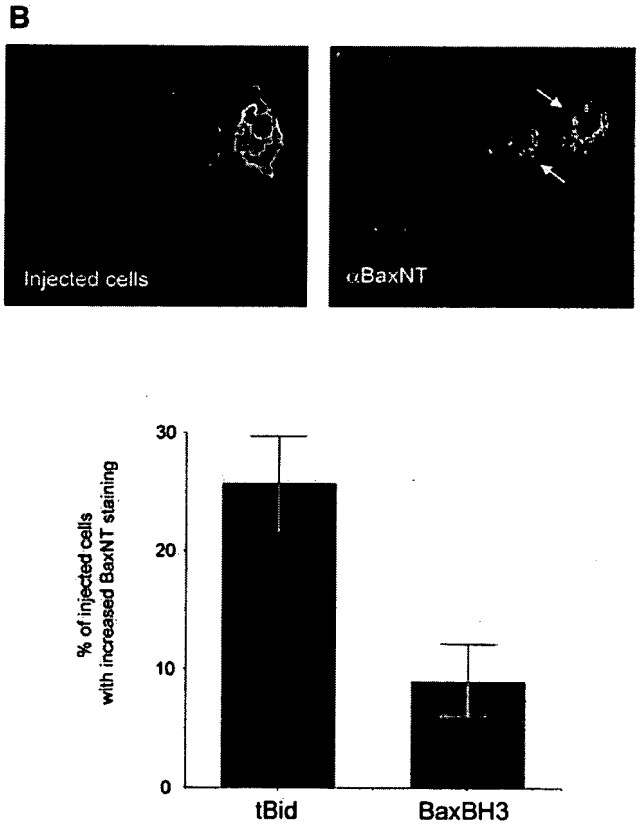


FIG. 2. **Effect of BaxBH3 on Bax mitochondrial targeting and conformation.** *A*, effect of BaxBH3 on Bax targeting to mitochondria. 4 fmol of IVTR Baxα were incubated with mitochondria in the absence or in the presence of the indicated concentrations of recombinant tBid or BaxBH3. The amount of radiolabeled Bax associated with the mitochondria was then analyzed by SDS-PAGE and autoradiography. Quantitative values were obtained as described under "Materials and Methods" and were normalized to the amount of Bax associated to mitochondria in the absence of tBid and BaxBH3. Data are means (±S.E.) of at least three independent experiments. *Top panel*, autoradiogram illustrating one representative experiment. *B*, effect of microinjected BaxBH3 on endogenous Bax conformation. BaxBH3 or tBid

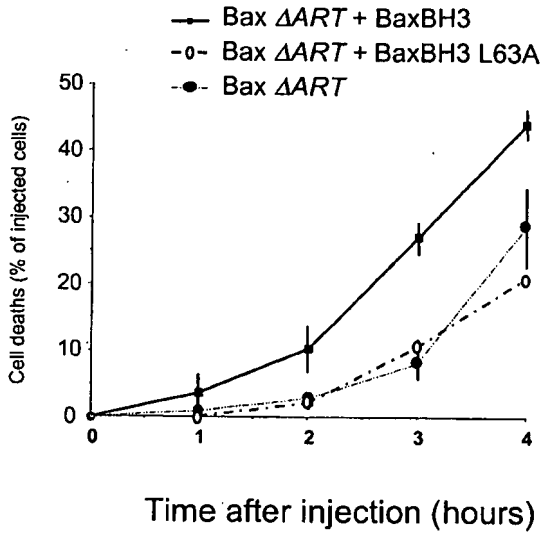


FIG. 3. **Effect of BaxBH3 on BaxΔART pro-apoptotic activity.** Recombinant BaxΔART (0.5 nM) alone or in the presence of either BaxBH3 (0.5 nM) or BaxBH3 L63A (0.5 nM) was mixed with Oregon Green dextran (0.5% w/v) in PBS and microinjected into Rat-1 fibroblasts, grown in 10% FCS. Cells were then incubated at 37 °C, and cell death in the microinjected population was assayed as in Fig. 1. Data are means (±S.E.) of three independent experiments.

color panel in Fig. 2*B*). Careful quantification indicated that ~25% of cells exhibited the change in Bax NT immunostaining 3 h after tBid microinjection (Fig. 2*B*). In sharp contrast, less than 10% of cells exhibited a similar feature 3 h after microinjection with BaxBH3 (0.5 nM). Thus, introduction of BaxBH3 into intact cells did not modify Bax conformation as efficiently as an equimolar amount of tBid. This suggests that BaxBH3 requires additional endogenous factors to induce efficiently a change in Bax conformation. Taken together, those results indicate that BaxBH3 is unable to induce a significant change in Bax conformation and to stimulate its association to mitochondria.

BaxBH3 Stimulates the Cytotoxic Activity of a Recombinant Bax Mutant Lacking Its N-terminal End—Although it is unable to stimulate Baxα directly, BaxBH3 nevertheless recruits Bax function in cells (24). One possible explanation for these paradoxical results is that BaxBH3 can functionally cooperate with Bax, provided Bax is in receipt of a sufficient threshold of activation by other signals. To address this question directly, we tested the effect of BaxBH3 on the pro-apoptotic function of a recombinant mutant form of Bax, BaxΔART. This recombinant protein consists of a Bax protein lacking its N-terminal 19 residues. This extreme N terminus of Bax normally regulates the apoptotic function of Bax negatively, and its deletion consequently results in an increased ability of Bax to homodimerize, interact with mitochondria, and promote apoptosis (25, 32, 36). Microinjection of recombinant BaxΔART (0.5 nM) induced

(0.5 nM) mixed with Oregon Green dextran (0.5% w/v) in PBS was microinjected into Rat-1 fibroblasts incubated in the presence of benzyloxycarbonyl-VAD-fluoromethyl ketone (50 μM). Cells were then incubated for 3 h at 37 °C prior to fixation and immunostained with anti-Bax NT antibody as described under "Material and Methods." Cells were then analyzed by fluorescence microscopy. The percentage of cells microinjected with either tBid or BaxBH3 that exhibits an increase in BaxNT staining was then analyzed. Data are means (±S.E.) of three independent microinjection experiments. *Top panel*, one representative example of Rat-1 fibroblasts microinjected with tBid. *Green* fluorescence allows identification of the microinjected cells, and *red* fluorescence shows BaxNT immunostaining of the same cells. *White arrows* indicate cells exhibiting a typical increase in Bax NT staining.

little apoptosis during the first 3 h following delivery (Fig. 3). However, by 4 h, a significant proportion of the microinjected cells exhibited morphological features of apoptosis. At this time, the extent of cell death observed in the microinjected population was 2-fold higher than that observed in control cells injected with the same amount of Baxα (compare Fig. 3 to Fig. 1*A*). This increased apoptosis in cells injected with BaxΔART, as opposed to cells injected with Baxα, was also observed at later time points (data not shown), indicating that, as expected, BaxΔART is more apoptogenic than Baxα. Co-injection of amounts of BaxBH3 that were unable to stimulate Baxα apoptotic activity (0.5 nM, see above in Fig. 1*B*) with BaxΔART led to a significant increase in the rate and the extent of cell death as early as 2 h following injection (Fig. 3). In sharp contrast, the mutant BaxBH3 L63A peptide had no effect on BaxΔART apoptotic activity (Fig. 3). Therefore, BaxBH3 can specifically sensitize cells to the deleterious effect of an activated form of Bax. This indicates that the inability of BaxBH3 to sensitize cells to Baxα relies on its inability to trigger a change in the conformation of the protein and not on its inability to sensitize cells to Bax apoptotic activity under the conditions used.

BaxBH3 Stimulates the Cytotoxic Activity of Wild Type Bax in the Presence of Bcl-xL—The observation that BaxBH3 can promote Bax-dependent apoptosis without providing a threshold for the activation of Baxα suggests that additional rate-limiting partners are required for BaxBH3 to promote Bax function. It is well established that BH3 peptides bind Bcl-xL, suggesting the possibility that BH3 peptides promote apoptosis by releasing Bax from dimers formed with its anti-apoptotic counterparts. Alternatively, additional factors, distinct from Bcl-xL itself, might instead be required for the peptide to activate Baxα indirectly. To distinguish between these two possibilities, we analyzed whether the presence of Bcl-xL suffices to allow our BH3 peptide to cooperate with Baxα.

We first analyzed the effect of BaxBH3 on mitochondrial targeting of Bax following its incubation with a recombinant GST-Bcl-xL fusion protein. As shown in Fig. 4*A*, IVTR Baxα preincubated with the control recombinant protein GST (2:1 ratio) interacted poorly with mitochondria whether or not BaxBH3 (200 nM) was present. IVTR Baxα preincubated with GST-Bcl-xL (2:1 ratio) also interacted poorly with mitochondria in the absence of BaxBH3. It should be noted that the recombinant Bcl-xL used here lacks its C-terminal end and is therefore unable to interact with mitochondria itself (24). Addition of 200 nM of BaxBH3 resulted in a significant increase in the mitochondrial targeting of IVTR Baxα preincubated with GST-Bcl-xL (Fig. 4*A*). Thus, the BH3 peptide can stimulate Baxα association with mitochondria provided Bcl-xL is present.

We next investigated whether the BaxBH3 peptide, when combined with Bcl-xL, elicits an increased ability of Baxα to induce cytochrome *c* release from isolated mitochondria. Incubation of isolated mitochondria (1.5 mg of protein/ml) with recombinant Baxα (5 nM) plus GST (2.5 nM) for 1 h at 30 °C led to no significant cytochrome *c* release from the mitochondrial pellet (data not shown). As well, the presence of an excess of BaxBH3 peptide (200 nM) plus Baxα/GST did not significantly affect cytochrome *c* release (Fig. 4*B*). In contrast, the addition of BaxBH3 (200 nM) to a combination of Baxα and GST-Bcl-xL in a 2:1 ratio leads to a significant loss of cytochrome *c* from mitochondria (Fig. 4*B*). Incubation of the BH3 peptide in the presence of GST-Bcl-xL had no effect on mitochondrial cytochrome *c* (data not shown), indicating that induction of cytochrome *c* release by BaxBH3 under the conditions used depends on the presence of both Baxα and Bcl-xL. Thus, BaxBH3 stimulates Baxα-induced cytochrome *c* release provided Bcl-xL is present.

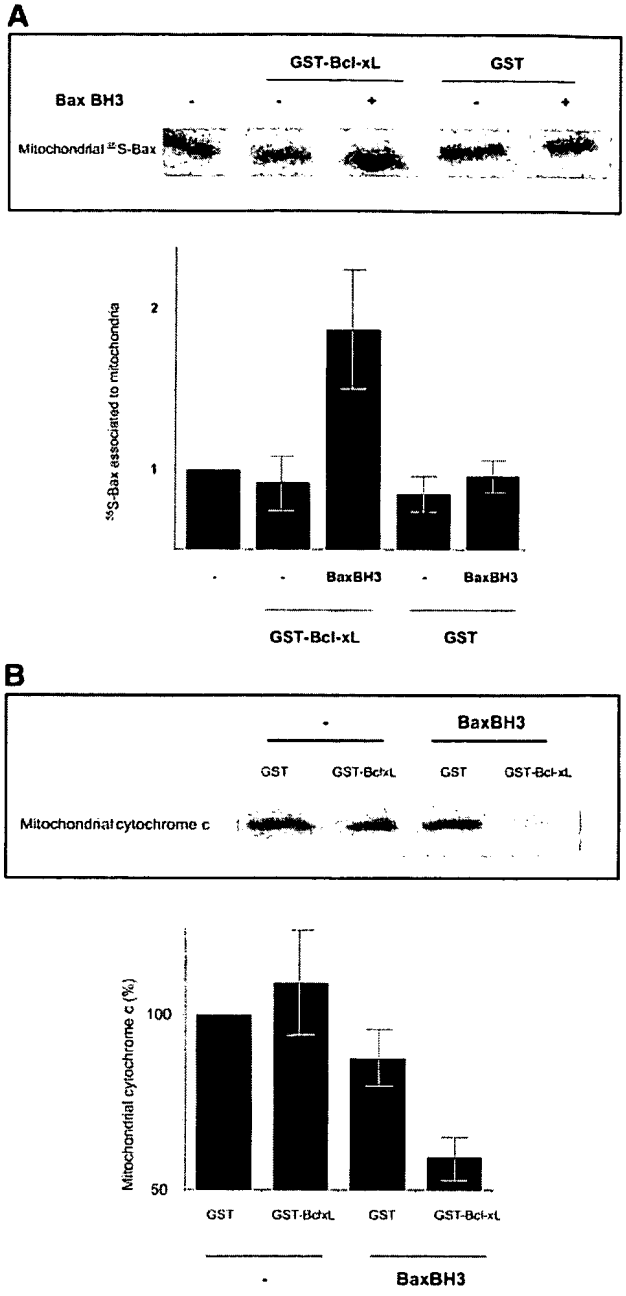


FIG. 4. **Effect of BaxBH3 on Bax interaction with mitochondria in the presence of Bcl-xL.** *A*, effect of BaxBH3 on Bax mitochondrial targeting in the presence of Bcl-xL. 4 fmol of IVTR Baxα were preincubated with 2 fmol of either GST-Bcl-xL or GST as a negative control prior to incubation with mitochondria (2.5 mg/ml) in the absence or in the presence of BaxBH3 (200 nM). The amount of radiolabeled Baxα associated to mitochondria was evaluated as described in Fig. 2*A*. Data are means (±S.E.) of four independent experiments. *Top panel*, autoradiogram illustrating one representative experiment. *B*, effect of BaxBH3 on Bax-induced cytochrome *c* release in the presence of Bcl-xL. Recombinant Baxα (5 nM) preincubated with either GST-Bcl-xL or GST (2.5 nM) was added to isolated mitochondria (1 mg/ml) in the absence or in the presence of BaxBH3 (200 nM) for 1 h at 30 °C. The amount of cytochrome *c* in the mitochondrial fraction was then evaluated as described under "Materials and Methods." The amount of mitochondrial cytochrome *c* following incubation with Baxα in the absence of Bcl-xL and BaxBH3 was used at 100%.

We next determined the ability of BaxBH3 to modulate Bax apoptotic activity in the presence GST-Bcl-xL. Baxα (0.5 nM) combined with GST-Bcl-xL (0.25 nM) was even less efficient in

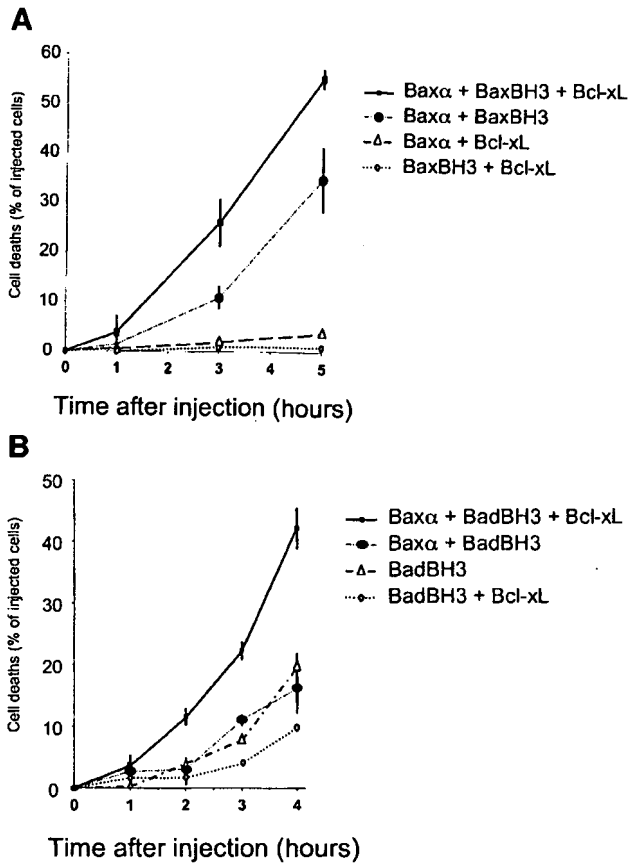


FIG. 5. **Effect of either BaxBH3 or BadBH3 on Bax pro-apoptotic activity in the presence of Bcl-xL.** *A*, the indicated combination of Baxα (0.5 nM), GST-Bcl-xL (0.25 nM), GST(0.25 nM), and BaxBH3 (0.5 nM) were mixed with Oregon Green dextran (0.5% w/v) in PBS and microinjected into Rat-1 fibroblasts, grown in 10% FCS. Cells were then incubated at 37 °C, and cell death in the microinjected population was assayed as in Fig. 1. Data are means (±S.E.) of three independent experiments. *B*, experiments were performed as in *A* using BadBH3 instead of BaxBH3.

inducing apoptosis than Baxα alone (compare Fig. 5*A* to Fig. 1, *top left panel*). This is consistent with the protective effect of microinjected GST-Bcl-xL against UV-induced apoptosis reported previously (24). This protective effect was further confirmed by the observation that BaxBH3 (0.5 nM) combined with GST-Bcl-xL was less efficient in inducing apoptosis than the peptide alone (compare Fig. 5*A* to Fig. 1, *bottom left panel*). In sharp contrast, addition of BaxBH3 (0.5 nM) to a mixture of Bax (0.5 nM) and GST-Bcl-xL (0.25 nM) led to a significantly more efficient induction of apoptosis than that observed in cells injected with BaxBH3 and Baxα in the absence of GST-Bcl-xL (Fig. 5*A*). This enhanced apoptosis was not observed when GST alone (0.25 nM) was used instead of GST-Bcl-xL, or when the mutant-inactive peptide BaxBH3 L63A (0.5 nM) was used instead of BaxBH3 (data not shown). Our recombinant Bcl-xL protein therefore diminishes the apoptotic activity of either BaxBH3 or Baxα alone, but it allows BaxBH3 to stimulate Baxα pro-apoptotic activity. In order to investigate whether this cooperativity between Bcl-xL and BaxBH3 can extend to other BH3 peptides, we used another synthetic 16-mer peptide encompassing the BH3 domain of Bad. It has been shown recently (22) that this BH3 domain is unable to activate Bax directly. Consistent with this observation, we observed that microinjection of BadBH3 (0.5 nM) alone or together with Baxα (0.5 nM) induced similar levels of apoptosis (Fig. 5*B*). BadBH3 (0.5 nM) combined with GST-Bcl-xL was less efficient in inducing apoptosis than the peptide alone (Fig. 5*B*). However, the addition of BadBH3 (0.5 nM) to a mixture of Bax (0.5 nM) and GST-Bcl-xL (0.25 nM) enhanced significantly the induction of apoptosis as compared with that observed with BadBH3 and Baxα in the absence of GST-Bcl-xL (Fig. 5*B*). Taken altogether, these data show that the presence of Bcl-xL is sufficient to allow both BaxBH3 and BadBH3 to stimulate the apoptotic activity of Baxα.

BaxBH3 Releases Bax from Heterodimers Formed with Bcl-xL—The previous results are mostly consistent with a model in which BaxBH3 releases an activated Bax from heterodimerization with Bcl-xL. To explore this idea, we analyzed the interaction between Baxα and Bcl-xL in the presence or in the absence of BaxBH3. We used a cell-free assay in which tagged recombinant proteins were assayed for their abilities to pull-down IVTR proteins (25). As shown in Fig. 6*A* (*left panel*), recombinant Baxα was unable to associate with either IVTR Baxα or IVTR BaxΔART as reported previously (25). In sharp contrast, recombinant Baxα associated with IVTR Bcl-xL under the same conditions (Fig. 6*A*, *left panel*). This binding was inhibited by an excess of BaxBH3 peptide but not by the inactive peptide BaxBH3 L63A (Fig. 6*A*, *left panel*). Conversely, recombinant Bcl-xL significantly bound to IVTR Baxα, in a manner that could be specifically inhibited by BaxBH3 (Fig. 6*A*, *right panel*). Of note, the amount of Baxα bound to recombinant Bcl-xL could be further increased by preincubating IVTR Baxα with unlabeled tBid (Fig. 6*A*, *right panel*). This suggests that the affinity of Bax for Bcl-xL correlates with its ability to adopt an active configuration, a notion that was confirmed by the finding that BaxΔART bound more efficiently to Bcl-xL than Baxα under similar conditions (Fig. 6*A*, *right panel*). Thus, Baxα is unable to homodimerize, but it nonetheless associates with Bcl-xL, albeit with less efficiency than its activated forms. Furthermore, BaxBH3 displaces Bax from the heterodimers it forms with Bcl-xL.

We then investigated whether the binding of Baxα to Bcl-xL, or its release from Bcl-xL upon treatment with BaxBH3, can significantly contribute to a change in its conformation and to its activation. In a first series of experiments, we analyzed the trypsin sensitivity of Bax following its association with Bcl-xL in cell-free pull-down assays. Previous experiments (28) have shown that incubation of Bax with apoptotic cytosols increases its resistance to mild proteolysis while concomitantly increasing its targeting to mitochondria. Moreover, mutants of Bax that exhibit an increased ability to homodimerize and to translocate to mitochondria also exhibit increased resistance to mild proteolysis (25). Consistent with the notion that a change in Bax sensitivity to proteolysis accompanies its activation, we observed that tBid-treated IVTR Baxα was highly resistant to mild proteolysis with trypsin, whereas untreated IVTR Baxα was not (Fig. 6*B*, *top panel*). In sharp contrast, Baxα sensitivity to proteolysis was neither affected by the presence of BaxBH3 nor by that of BaxBH3L63 (Fig. 6*B*, *top panel*). In a pull-down experiment using recombinant His-tagged Bcl-xL as described above, most Bcl-xL-bound IVTR Baxα exhibited resistance to trypsin treatment, whereas there was no detectable trypsin-resistant Baxα in the unbound fraction (compare Fig. 6*B*, *bottom panel*, to Fig. 6*A*). Addition of BaxBH3 resulted in a complete loss of Bcl-xL-bound, protease-resistant Baxα and the concomitant appearance of protease-resistant Baxα in the unbound fraction (Fig. 6*B*, *bottom panel*). In control experiments, no effect of the mutant BaxBH3 L63A was observed. Thus, Bcl-xL binding to Baxα is sufficient to modify its sensitivity to proteolysis, whereas the action of BaxBH3 is to trigger the release of a modified Bax from Bcl-xL. In a second series of

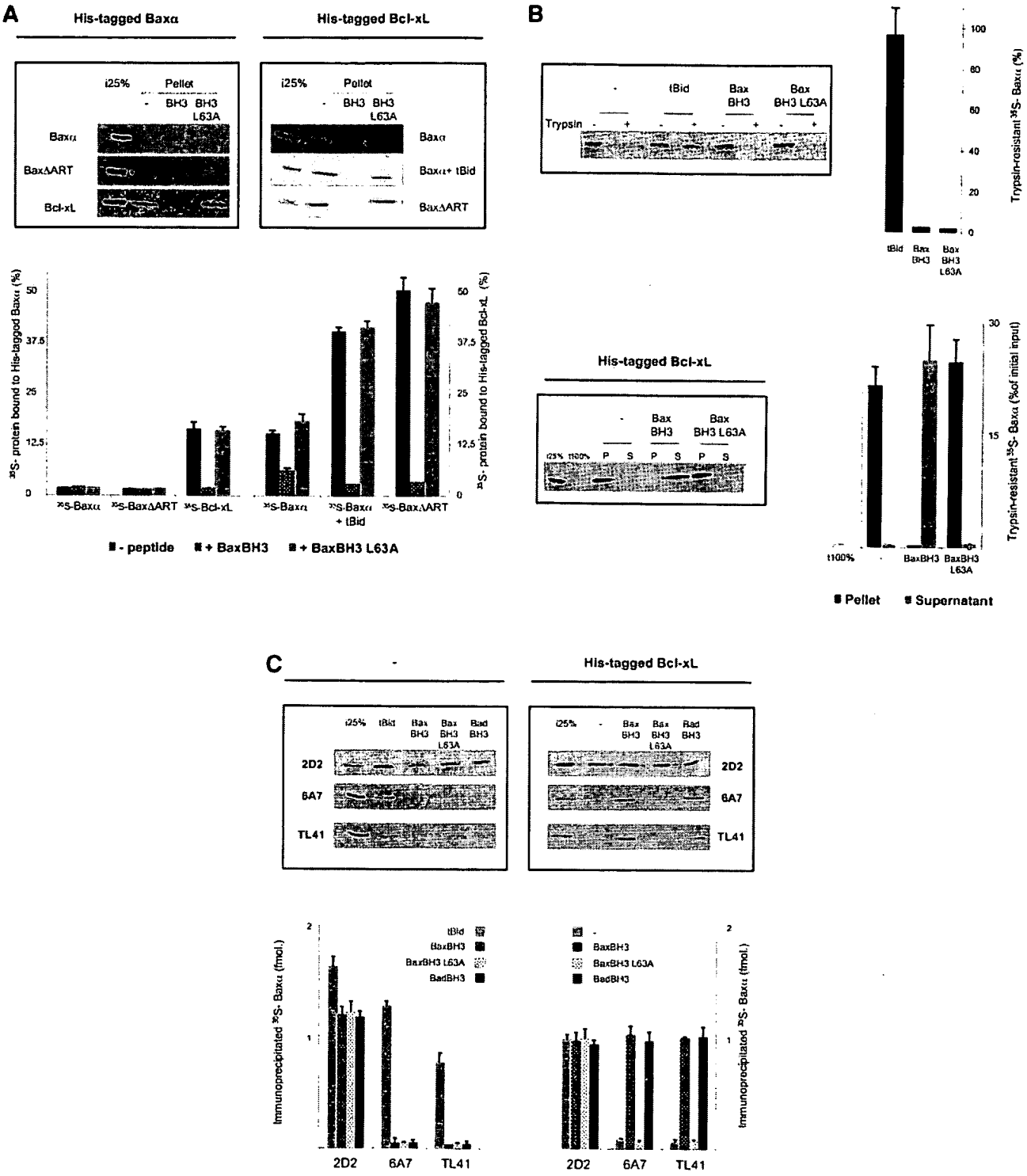


FIG. 6. **Effect of either BaxBH3 or BadBH3 on Bax/Bcl-xL interactions *in vitro*.** *A, left panel*, IVTR Baxα, BaxΔART, or Bcl-xL (4 fmol) were preincubated with purified His-tagged Baxα before addition of the indicated peptides (200 nM final concentration). The percentage of ^{35}S-labeled proteins bound to His-tagged Baxα was estimated after SDS-PAGE and fluorography using the initial input (4 fmol) as 100% as described under "Materials and Methods." Data are means (±S.E.) of three independent experiments. *Top panel*, autoradiogram illustrating one representative experiment. 1 fmol of each radiolabeled protein (i25%) was loaded where indicated. *Right panel*, IVTR Baxα (4 fmol in the absence or in the presence of 4 fmol of unlabeled tBid) or BaxΔART (4 fmol) was preincubated with purified His-tagged Bcl-xL before addition of the indicated peptides (200 nM final concentration). The percentage of ^{35}S-labeled proteins bound to His-tagged Bcl-xL was evaluated as described above. Data are means (±S.E.) of three independent experiments. *Top panel*, autoradiogram illustrating one representative experiment. *B, top panel*, IVTR Baxα (8 fmol) preincubated with either tBid (0.2 nM) or with the indicated peptides (200 nM) was treated or not with trypsin as described under "Materials and Methods." The amount of protease-resistant ^{35}S-labeled proteins was evaluated and expressed as a fraction of the initial amount of radiolabeled Bax. Data are means (±S.E.) of three independent experiments. *Top panel*, autoradiogram illustrating one representative experiment. *Bottom panel*, IVTR Baxα (8 fmol) was preincubated with purified His-tagged Bcl-xL in the presence of the indicated peptides (200 nM) as in *A* (*right panel*). His-bound complexes (pellet, *P*) and free proteins (supernatant, *S*) were then treated with trypsin as described under "Materials and Methods." The amount of protease-resistant ^{35}S-labeled proteins was then evaluated and expressed as a fraction

experiments, we analyzed the ability of three epitope-specific antibodies to immunoreact with Bax in the presence of Bcl-xL, BH3 peptides, or both. The specific anti-Bax antibodies used were as follows: (i) the 2D2 monoclonal antibody that was raised against a peptide encompassing residues 3–16 of human Bax, an epitope which is exposed regardless of Bax conformation (37); (ii) the 6A7 monoclonal antibody that was raised against a peptide encompassing residues 12–24, which only binds to active Bax (38); (iii) the TL41 polyclonal antibody that was produced against a peptide sequence (residues 57–72), which represents the minimal Bax BH3 domain (24). In non-denaturing conditions, only the 2D2 antibody could immunoprecipitate IVTR Baxα that was left untreated (data not shown) or that was treated with the control peptide BaxBH3 L63A (Fig. 6*C*, *left panel*). In sharp contrast, all three antibodies could immunoprecipitate t-Bid treated IVTR Baxα (Fig. 6*C*, *left panel*). This indicates that the epitope for the 6A7 antibody, and the epitope for the TL41 antibody within the Bax BH3 domain, are normally hidden in native Bax but become exposed following activation of Bax by tBid. Treatment of IVTR Baxα with either BaxBH3 or BadBH3 had no effect on the immunoreactivity of the radiolabeled protein with either the 6A7 or the TL41 antibodies (Fig. 6*C*, *left panel*), further supporting the notion that these peptides cannot activate Bax by themselves. As shown in Fig. 6*C* (*right panel*), radiolabeled Bax complexed to Bcl-xL could be immunoprecipitated by the 2D2 antibody. This observation is consistent with the ability of an antibody raised against the equivalent peptide sequence to immunoreact with Bcl-xL-bound murine Bax reported previously (38). In sharp contrast, neither the 6A7 nor the TL41 conformation-specific antibodies could immunoprecipitate Bcl-xL-bound IVTR Baxα in native conditions (Fig. 6*C*), although they could do so in denaturing conditions (0.1% SDS, 0.1% Triton X-100, data not shown). Thus, the inability of these antibodies to immunoreact with Bax in non-denaturing conditions likely results from the inaccessibility of the corresponding epitopes in the Bcl-xL-bound protein. Moreover, when Bcl-xL-Bax complexes were treated with either BaxBH3 or BadBH3, but not with the mutant peptide BaxBH3 L63A, Baxα was significantly immunoprecipitated by the 2D2, the 6A7, and the TL41 antibodies (Fig. 6*C*, *right panel*). Similarly, IVTR Baxα directly released from nickel-Sepharose-bound His-tagged Bcl-xL by wild type BH3 peptides (as in Fig. 6*B*) also immunoreacted with all three antibodies (data not shown). Thus, both epitopes for the 6A7 antibody and the TL41 antibody become accessible when Bax molecules are specifically released from Bcl-xL by BH3 peptides. Taken altogether, these results indicate that, when it is directly activated by tBid, Bax becomes resistant to proteolysis, whereas its BH3 domain and a region in its N-terminal end (the 6A7 epitope) become exposed. Binding to Bcl-xL is sufficient to modify the sensitivity to proteolysis of Bax, but the release of Bax from Bcl-xL, induced by BH3 peptides, is necessary to produce an active Bax, as probed by the accessibility of the resulting protein to conformation-specific antibodies.

DISCUSSION

The underlying mechanism by which BH3-only proteins recruit Bax and Bak to induce apoptosis is a key to the way mitochondria integrate multiple stimuli into one single apoptotic pathway. In many healthy cells, Bax and Bak appear to be expressed in an inactive conformation, unable to self-associate. Thus, to exert their apoptotic function, these proteins need to undergo conformational changes. Emerging evidence indicates that some BH3-only proteins, such as Bid, can function as death agonists that activate directly Bax and Bak. It is formally possible that certain domains in those death agonists, distinct from the BH3 one, could contribute to their ability to promote the pro-apoptotic changes in Bax or Bak. However, the ability of these proteins to activate Bax/Bak seems to rely in great part on some specific information contained in their BH3 domain. Indeed, short synthetic peptides encompassing certain BH3 domains are sufficient to trigger Bax/Bak activation (22).

In this study, we used a synthetic peptide representing the BH3 domain of Bax (BaxBH3), which composes the minimal sequence required to antagonize anti-apoptotic family members. We report here that BaxBH3 has no significant effect on either the apoptotic activity, the mitochondrial targeting, and/or the conformation of Baxα (Figs. 1 and 2). We found that another synthetic peptide representing the minimal BH3 domain of Bad is also unable to stimulate Bax activity (Fig. 5*B*). Thus, BH3 peptides that contain sufficient information to occupy the surface pocket of the anti-apoptotic Bcl-2 family members do not all necessarily support direct activation of Bax/Bak. Consistent with this, BH3 peptides derived from Bad, Bik, or Noxa were shown to lack the ability to activate Bax/Bak (22), whereas small molecule non-peptidic ligands of Bcl-xL are reportedly unable to induce Bax insertion into mitochondrial membranes (6). These observations show that another subset of BH3 domains exists, which can function as survival antagonists but not as death agonists for Bax/Bak.

The lack of effect of BaxBH3 on Baxα activity sharply contrasts with the ability of BaxBH3 to promote the apoptotic function of BaxΔART, an N-terminal deletion of Bax that displays an enhanced ability to homodimerize and to induce apoptosis (Fig. 3). The fact that the activity of BaxΔART can be further enhanced by BaxBH3 indicates that BaxBH3 can functionally cooperate with Bax, so long as Bax is provided with a sufficient trigger to lower its threshold of activation. Because BaxBH3 prevents BaxΔART binding to Bcl-xL (Fig. 6*A*), BaxBH3 is likely to enhance the apoptotic activity of BaxΔART by preventing the inhibitory association of this activated form of Bax with endogenous Bcl-xL and possibly Bcl-2. One interesting implication from this is that death agonists and survival antagonists (typified by our BH3 peptide) may activate cell death by mechanistically distinct, yet cooperative pathways. In agreement with this, we have observed that BH3 peptides can sensitize cells to apoptosis induction by microinjected tBid.[2]

Even though BaxBH3 is unable to activate Bax directly, the fact remains that it induces apoptosis (Fig. 1; see also Refs. 39 and 40). Our experiments therefore constitute proof of concept that the ability to function as a death ligand is not absolutely required for a BH3 domain to promote apoptosis. The observation that the BH3-only protein BimL lacks the ability to acti-

[2] C. Moreau, P.-F. Cartron, A. Hunt, K. Meflah, D. R. Green, G. Evan, F. M. Vallette, and P. Juin, unpublished data.

of the initial amount (8 fmol) of radiolabeled Bax. Data are means (±S.E.) of four independent experiments. *Top panel*, autoradiogram illustrating one representative experiment. 2 fmol of untreated radiolabeled Bax (25%) and 8 fmol of trypsin-treated radiolabeled Bax (t100%) were loaded where indicated. *C*, IVTR Baxα (4 fmol, *left panel*) or an equivalent amount of His-tagged Bcl-xL-bound IVTR Bax (prepared as described under "Materials and Methods," *right panel*) were incubated, as indicated, with either tBid (0.2 nM), BaxBH3 (200 nM), BaxBH3 L63A (200 nM), BadBH3 (200 nM), or left untreated prior to immunoprecipitation with the indicated antibodies. Immunoprecipitated ^{35}S-labeled proteins were analyzed and quantified as described under "Materials and Methods." Data are means (±S.E.) of three independent experiments. *Insets*, autoradiograms illustrating one representative experiment for each condition. 1 fmol of the initial, non-immunoprecipitated radiolabeled Bax (25%) was loaded in each gel for illustrative purposes.

vate Bax directly but retains the ability to interact with Bcl-xL and induce apoptosis is consistent with this view (41). As BaxBH3 specifically induces apoptosis by a mechanism requiring the presence of either Bax or Bak (24), these experiments also imply that the ability of this peptide to promote Bax/Bak-mediated apoptosis must involve some other intermediaries. One possibility is that survival antagonists such as BaxBH3 promote the release from Bcl-xL/Bcl-2 of some death agonists such as Bid (17). It should be noted, however, that a mutant of Bcl-xL that cannot interact with Bax or Bak but interacts with Bid is less efficient than wild type Bcl-xL in preventing Bax-mediated mitochondrial dysfunction (42). Thus anti-apoptotic Bcl-2 family members may act on Bax/Bak directly, and their functional antagonists may promote Bax/Bak activity by suppressing this interaction. We found that addition of Bcl-xL was sufficient to allow BaxBH3 to stimulate the ability of Bax to interact with mitochondria, to induce cytochrome *c* release (Fig. 4), and to induce apoptosis upon microinjection (Fig. 5*A*). Similarly, we found that BadBH3 had no impact on the apoptotic activity of microinjected Bax by itself but that it could stimulate it when combined with Bcl-xL (Fig. 5*B*). Thus, induction of Bax-mediated apoptosis by a BH3 domain does not necessarily involve any other partner than Bcl-xL itself. This strongly favors a model in which survival antagonists promote apoptosis by inducing the release of Bax/Bak from heterodimers they engage with Bcl-xL/Bcl-2.

Most of the arguments against the aforementioned model arise from the observation that Bax/Bak and their anti-apoptotic counterparts exhibit only modest interaction in some cell types unless apoptosis is triggered (38, 43). We confirm that Bax binds to Bcl-xL with greater efficacy in its active conformation; its association to Bcl-xL is indeed stabilized by the addition of tBid or by a deletion in its N-terminal regulating domain (Fig. 6). However, we have also observed that, despite its inability to self-associate, Baxα can bind to Bcl-xL (Fig. 6). This observation is consistent with whole cells studies in which Bax fused to a fluorescent protein, although inefficient at inducing apoptosis by itself, nonetheless exhibited significant interaction with Bcl-2 and Bcl-xL as assayed by fluorescence resonance energy transfer (6, 44).

Baxα/Bcl-xL interactions were significantly inhibited by BaxBH3 (Fig. 4). Similarly, *in vivo* interactions between Bax and Bcl-xL were disrupted by small molecule ligands of the BH3-binding pocket of Bcl-xL (6). Thus, binding of the BH3 domain of Bax within the hydrophobic pocket of Bcl-xL is likely to be essential for the formation of a stable heterodimer. Residues within the BH3 domain of Bax that are critical for dimer formation are oriented toward the hydrophobic core of wild type Bax (46). Thus, major conformational modifications in Bax should accompany its BH3-dependent binding to Bcl-xL. Our observation that, upon its binding to Bcl-xL, Bax acquires resistance to mild proteolysis is consistent with this idea (Fig. 6). It is striking to note that protease resistance is a property that Bax also displays when it is activated by apoptotic cytosols (28), tBid (this study), or by site-directed mutagenesis (25). Thus, although the very nature of the protease-resistant, Bcl-xL-bound form of Bax requires further characterization, it is tempting to speculate that it may be close, on some aspects, to that of an active Bax. Microinjection experiments nevertheless suggest that, as long as it is bound to Bcl-xL, Bax does not exert its apoptotic activity (Fig. 5). Our immunoprecipitation experiments using conformation-specific antibodies further confirms this by showing that Bcl-xL-bound Bax does not fulfill all the criteria of an active Bax (Fig. 6). Indeed, tBid-activated Bax, but not native or Bcl-xL-bound Bax, can immunoreact with the well documented conformation-specific 6A7 antibody and with a polyclonal antibody raised against the minimal Bax BH3 domain (TL41). It was initially proposed that the epitope for the 6A7 antibody might be in the vicinity of the BH3 dimerization domain of Bax (38). Our observation that exposure of the 6A7 epitope occurs concomitantly with the exposure of an epitope within the BH3 domain of Bax is certainly consistent with this view. It also indicates that the BH3 domain of Bax is hidden within the native protein but is exposed when Bax is active. Dimerization of Bax with Bcl-xL may prevent both the 6A7 and the TL41 epitopes from being exposed, or alternatively, it may mask these epitopes on Bax (38). When specifically released from Bcl-xL by BH3 peptides, however, Bax recapitulates the features of tBid-activated Bax, as it becomes accessible to both conformation-specific antibodies and retains its resistance to mild proteolysis. This is most consistent with a model in which Bcl-xL binds to inactive Bax, modifies it, and then releases active Bax in response to a BH3 peptide. One provocative implication from this is that Bcl-xL should cooperate with ligands of its BH3 domain-binding pocket to induce Bax-mediated apoptosis, which may explain why cells overexpressing Bcl-xL are more sensitive to induction of apoptosis by a BH3 mimetic (47).

In summary, we have analyzed in this study the apoptotic function of a synthetic BH3 peptide comprising sufficient information to antagonize functionally anti-apoptotic Bcl-2 family members, and we found that it does not function as a death agonist of Bax. This BH3 peptide can nonetheless promote Bax-dependent apoptosis by interfering with the ability of Bax to interact with, and be suppressed by, Bcl-xL. There is a growing list of small molecules that have been selected for their ability to occupy the BH3-binding pocket of anti-apoptotic Bcl-2 family members and that act as true BH3 mimetics (6, 45, 47). Our results indicate that such molecules may efficiently induce apoptosis in cells expressing high levels of the anti-apoptotic Bcl-2 family members by triggering the release of active multidomain pro-apoptotic proteins from these survival proteins.

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